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STUDY ON TUMOR BIOLOGY AND THE AGING EFFECT OF CHEMOTHERAPEUTIC TREATMENT IN OLDER BREAST CANCER PATIENTS

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Omdat verwezenlijkingen van één persoon het resultaat zijn van vele sterke schouders om op te staan.

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List of abbreviations

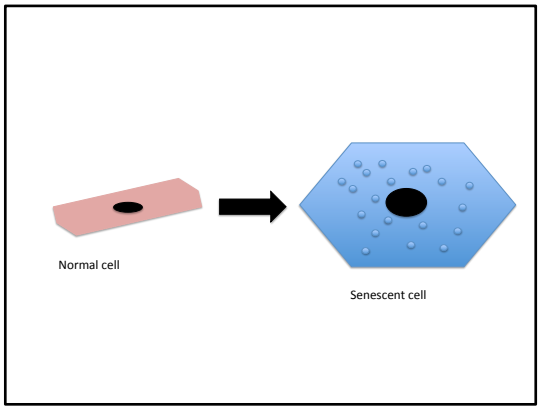
ADL	Activities of daily living
ARF	Alternative Reading Frame
AST	Autophagy to senescence transition
ATG16L1	Autophagy Related 16 Like 1
BMI	Body Mass Index
BNIP3	Bcl2/adenovirus E1B 19 kDa Interacting Protein 3
CAF	Carcinoma Associated Fibroblast
CAV-1	Caveolin-1
CCI	Charlson Comorbidity Index
CDK	Cyclin dependent kinase
(C)GA	(Comprehensive) geriatric assessment
CMV	Cytomegalovirus
CRAMP	Cathelicidin-Related Antimicrobial Peptide
CRASH	Chemotherapy Risk Assessment Scale for High Age Patients
CRP	C-reactief Proteine
CTSB	Cathepsine B
ECOG-PS	Eastern Cooperative Oncology Group Performance Status
EF-1α	Elongation Factor alpha
ES	Enrichment Score
fTRST	Flemisch version of the Triage Risk Screening Tool
FISH	Fluorescence In Situ Hybridisation
GDS	Geriatric Despression Scale
GEO	Gene expression Omnibus
GFI	Groningen Frailty Indicator
GRO	Groucho (=C-X-C-motif chemokine ligand)
GSEA	Gene Set Enrichment Analysis
H&E	Hematoxilin – Eosin
HGF	Hepatocyte Growth Factor
HR	Hazard Ratio
iADL	instrumental activities of daily living
ICAM	Intercellular Adhesion Molecule 1
IGF-1	Insulin-like Growth Factor – 1
IGFBP	Insulin-like Growth Factor Binding Protein
IL-	Interleukin-
IRP	Immune Risk Profile
ISS	Insulin/insulin-like growth factor signaling pathway
LCM	Laser Capture Microdissection
LDH	Lactacte Dehydrogenase
LOFS	Leuven Oncogeriatric Frailty Score
(L)TL	(Leukocyte) Telomere Length
MCP-1	Monocyte Chemotactic Protein – 1
MDM2	Mouse Double Minute 2 homolog
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinase
MMSE	Mini Mental State Examination
MNA-sf	Mini Nutritional Assessment – short form
PTEN	Phosphatase and Tensin homolog
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RB	Retinoblastoma
RQI	RNA Quality Indicator
RT-qPCR	Real Time – quantitative Polymerase Chain Reaction
SASP	Senescence associated secretory profile
SIOG	International Society of Geriatric Oncology
TNF-α	Tumor Necrosis Factor alpha
TRF	Telomere Restriction Fragment
TRST	Triage Risk Screening Tool
TP53	Tumor Protein 53
TUG	Timed Up and Go
uPAR	urokinase type Plasminogen Activator Receptor
UPR	Unfolded protein response
VEGFA	Vascular Endothelial Growth Factor A
VES-13	Vulnerable Elderly Survey-13
µ	Micro

1. The paradox: senescence protects against cancer, and causes cancer
    a. Cellular senescence – aging and cancer

The biological process of aging is a complex mechanism. It is not reducible to a single physiological change in the organism, but it concerns a multifactorial process. A lot of research has already been performed on the topic, and various physiological age-related changes have been identified.

Cellular senescene is thought to represent one of the capital molecular processes in biological aging. It serves primarily as a protection mechanism that shuts down damaged cells. They are forced into a state of irreversible growth arrest[1,2]. Senescent cells are characterized by a specific phenotype (enlarged size, flattened morphology, senescence associated β-galactosidase activity, reorganization of chromatin into foci of herterochromatin and resistance to apoptosis)[3] (Fig 1).

A



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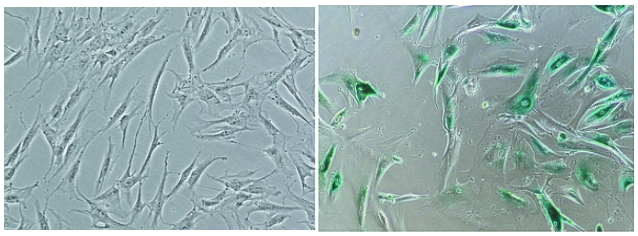

Figure 1: (A) Schematic representation of the transition of a normal cell to a senescent cell, (B) microscopic features of normal cells versus senescent cells after staining for β-galactosidase activity (www.sigmaaldrich.com; catalogue#CS0030)

Triggers that induce the senescence program are various: telomere erosion, unresolved DNA damage, lysosomal stress, unresolved UPR (unfolded protein response), oncogene activation, culture shock or reactive oxygen species[4]. The induction of senescence in a damaged cell protects the organism from developing cancer, as it is characterized by unability to re-enter the cell division cycle in response to mitogens and by an acquired resistance to oncogenic stimulation. Thereby, it prevents damaged cells from uncontrolled proliferation and dissemination. Senescence is therefore believed to be an evolutionary selected mechanism that preserves the integrity of the young organism during reproductive lifespan (Fig 2).

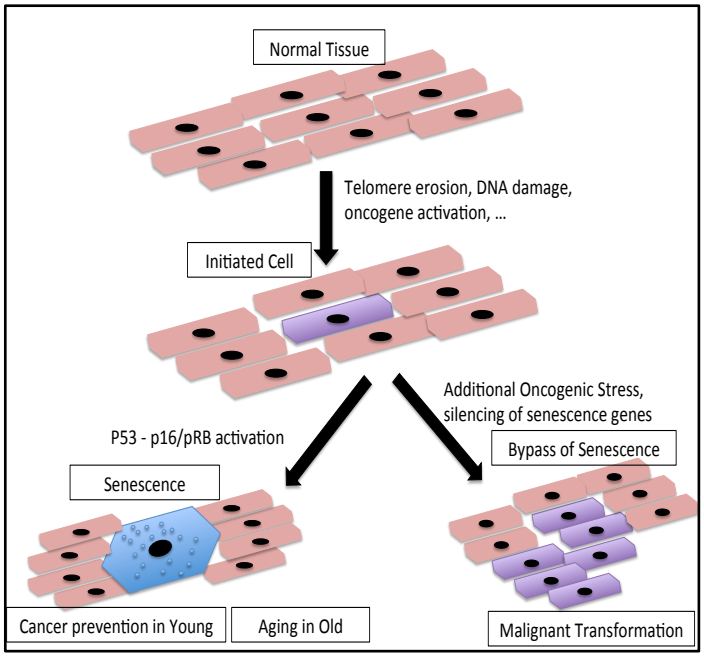

Figure 2: Adapted from Shan et all [5]
<u>The two-sided face of senescence: protection against cancer and aging.</u>
Normal tissue cells can be initiated towards a preneoplastic state by several triggers (telomere erosion, DNA damage, oncogene activation, …). Activation of TP53, p16, and RB gene induce senescence in this cell, preventing it from turning into a cancerous lesion. Whereas this mechanism protects from cancer in young life, it causes aging in older life. If this mechanism of senescence is bypassed through additional (epi-)genetic changes, preneoplastic cells can evolve towards a malignant tumor.

Senescence is induced through upregulation of several senescence genes, the most robust genes being CDKN2A (p16/INK4A/ARF), TP53, RB (Retinoblastoma gene). A fourth gene, CDKN1A (p21/WAF1/CIP1), has also a role in inducing growth arrest, but is a less reliable senescence marker, as the growth arrest induced by upregulation of this gene can be more transient[4].
The pathway by which these genes induce cell cycle arrest is depicted in figure 3, taken from one of our own publications[6].
CDKN2A is a complex gene that encodes two distinct proteins, $p16^{INK4a}$ and $p14^{ARF}$. Despite arising from the same gene, there is no protein sequence similarity between these products. The locus has a complex architecture, containing two separate promoters that generate transcripts with different first exons followed by common second and third exons. Because the shared exons are read in different reading frames they are not isoforms and have no amino acid homology. $p16^{INK4a}$ is encoded by

exons 1α, 2, and 3. It functions as a cyclin-dependent kinase inhibitor of the cell cycle by inhibiting the activity of the cyclin-dependent kinase complex "cyclin D/CDK4/CDK6", thereby inhibiting the pRB phosphorylation and blocking the passage from G_1 into S [7,8]. The alternate reading frame product, $p14^{ARF}$, is encoded by a different first exon (exon 1β) that is upstream of exon 1α, using the same second exon as $p16^{INK4a}$ but in a different reading frame[8]. The amino-acid coding sequence of $p14^{ARF}$ ends in exon 2, with the remainder of exon 2 and exon 3 comprising the 3'-untranslated region[9]. $p14^{ARF}$ functions by preventing p53 degradation, thereby allowing p53-mediated apoptosis or cell cycle arrest.

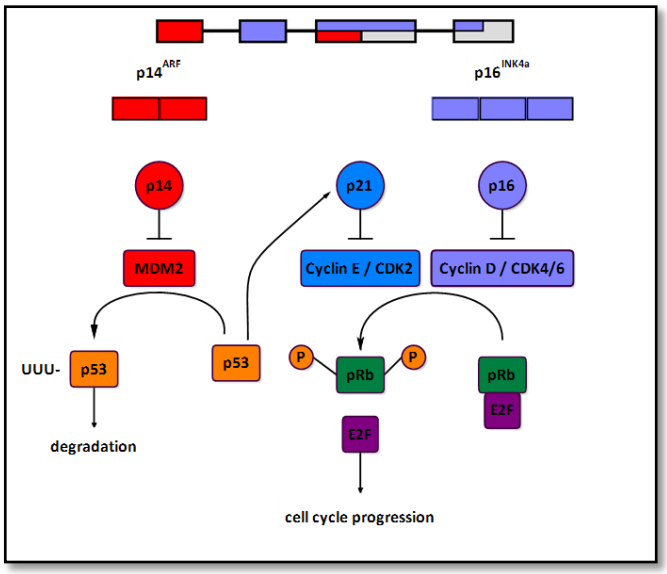

Figure 3: Reprinted with permission from Elsevier.
<u>The p16 locus and cell cycle control.</u>
The p16 locus encodes 2 overlapping proteins, p16 and ARF, by using different first exons and common second and third exons. These structurally very different proteins both act as negative regulators of the cell cycle, p16 inhibits the activation of CDK4 and CDK6 by cyclin D, hence preventing subsequent phosphorylation of pRB and thus cell cycle progression. ARF regulates p53 activity by binding with MDM2, an ubiquitin ligase that otherwise targets p53 for its degradation by proteasome. High levels of ARF stabilize p53 permitting it to induce p21, a cell cycle inhibitor that blocks CDK2/cyclin E – mediated phosphorylation of pRB.

Lately, also PTEN has come across as a potential candidate gene involved in senescence[10]. PTEN is an established tumor suppressor gene. Very often it is mutated in human tumors[11]. Recent transgenic mouse models have highlighted a role in the aging process as well[12,13]. The two mouse models display systemic PTEN overexpression, but under normal regulatory control. These mice exhibit, next to reduced adiposity and metabolic changes, higher median and maximal lifespans, independent of the tumor suppressor function of PTEN.
In the past, downregulation of the nutrient sensing IIS (Insulin/insulin-like growth factor signaling) pathway has been shown to be a main modulator of longevity conserved across evolution (cfr lifespan extension of organisms through caloric restriction). The observation that PTEN overexpression in mice extends their lifespan, adds further evidence to this paradigm

However, there seems to be a price to pay for this protection mechanism. In exchange for organismal integrity in younger life, accumulation of senescent cells throughout the body causes biological aging in older life. According to a current hypothesis, which originated from the finding that senescent cells accumulate in vitro with increasing population doublings until the majority of the culture has reached replicative senescence, senescent cells accumulate in the organism and due to their lack of regenerative capacity, this results in failure of organ homeostasis and function and, consequently, tissue aging[14]. Senescent cells have been reported in vivo, in a variety of tissues of different organisms including mouse, primates and humans[15-19]. Also have there been studies providing evidence that increasing age does result in a higher frequency of senescent cells[15-17,20], be it mostly in skin. The identification of signs of senescence at specific sites of age-related pathologies, further suggests the link between cellular senescence and aging[21-24].

The dual role of senescence genes (Fig 2) in aging and cancer is further illustrated by progeroid syndromes (e.g. Werner's syndrome, Hutchinson-Gilford progeria syndrome). These are characterized by defects in DNA repair mechanisms. As the naturally occurring DNA damage in these patients is not repaired correctly, the patients develop severe aging sings at young age, because of widespread activation of senescence and/or apoptosis in damaged cells. Some of these syndromes, like Werner's syndrome, are also characterized by high risk of developing cancer at young age.

Furthermore, the trade-off between cancer and aging has been nicely illustrated by impressive mice-experiments, where TP53 was manipulated in order to observe the effects on the aging process and the development of cancer: mice with one knock-out allele of TP53, died mostly because of cancer. If however, they happened to escape from cancer, they displayed a longer lifespan than normal counterparts, showing that decreased occurrence of senescence restrains aging. Mice transfected with a constitutively active allele of TP53, had a greatly reduced cancer incidence, but showed premature aging. If mice were armed with an extra allele of TP53, but under normal control (so not constitutively activated), they did not show this enhanced aging phenotype, but did however have improved tumor clearance.[25-29]

b. Tissue aging

The process of molecular senescence is a general concept potentially occurring in every cell type. Nevertheless, some tissues or organs seem more prone to accumulate senescent cells, and others do only contain sporadic senescent cells even after many years of age.

Haematologic progenitor cells for example, do have a hughe mitotic activity throughout life, as they have to repopulate the blood as differentiated blood cells reach the end of their lifespan or expand the population of circulating lymphocytes when confronted with new or known antigens.
Circulating white blood cells represent easily accessible cells to measure the reflection of the aging process on the blood forming organs. They are quite particular as they are freely circulating and not fixed in a surrounding structure like other organs e.g. the breast gland, gastro-intestinal organs, and many others.
In the breast, the functional glandular and ductal elements are embedded in fibrofatty tissue that forms the bulk of the mammary gland. The proportions of fat and collegenous stroma vary among individuals. The majority of cells that form the duct epithelium are columnar or cuboidal cells lining the lumen. Myoepithelial cells lie between the epithelial layer and the basal lamina. The normal periductal stroma contains fibroblasts, elastic and collageneous fibers, a scattering of lymphocytes (scarce in normal conditions), plasma cells, mast cells and histiocytes (Fig 4).

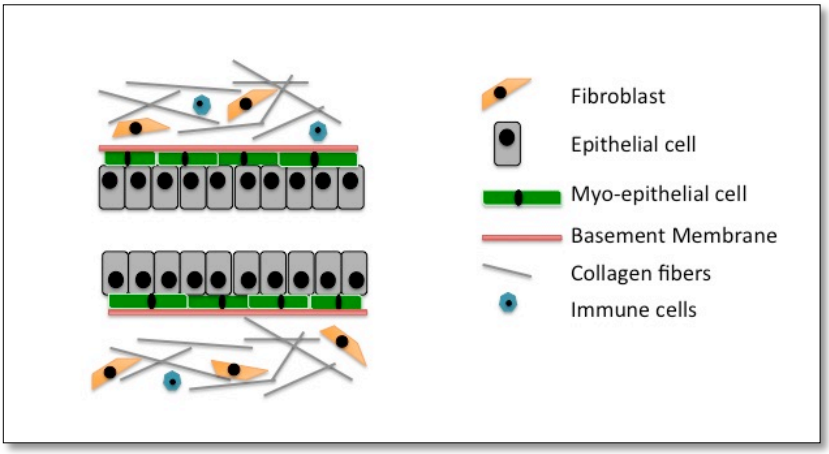

Figure 4: Composition of the normal breast epithelium and stroma

Senescence can occur in epithelial cells, fibroblasts or other cell types. High frequency of senescence in epithelial cells or fibroblasts of the tissue stroma will in the first place result in an aging phenotype of the affected organ.

c. <u>The senescence associated secretory profile</u>

In the first paragraph, we explained the role of senescence in aging and cancer prevention, and the trade-off that exists between both.
The complexity of the interaction aging-cancer grows by the fact that senescence by itself can be stimulatory on the occurrence of cancer. This theory is called the theory of Antagonistic Pleiotropy. By natural selection, senescence is primarily a protection mechanism. Once the pressure of natural

selection declines (after the reproductive period), detrimental mechanisms are no longer eradicated as efficiently as before. This could be the explanation why senescence, protecting from cancer in younger age, seems to show cancer-promoting effects on the longer term.

Cells that have activated the senescence program are arrested in cell cycle phase G0. As described previously, they acquire a specific phenotype with an enlarged flattened morphology, senescence associated β-galactosidase activity, reorganization of chromatin into foci of herterochromatin and resistance to apoptosis. But they keep an active metabolism, and acquire a Senescence Associated Secretory Phenotype (SASP)[3,30]. This SASP is composed of matrix remodeling enzymes, inflammatory mediators, angiogenic factors and growth factors, that are produced by the senescent cell itself, with the purpose to signal in a paracrine way it's compromised status to the cells around. The purpose of the SASP is thought to be dual: retaining the permanent growth arrest, while attracting immune cells to the damaged cell in an attempt to destroy it. Nevertheless, it has been observed that meanwhile this SASP has harmful effects on the cells surrounding the cell of origin.

As described in the previous paragraph, most organs or tissues in the body are not solely composed of a single cell type, but consist of epithelial cells and surrounding stroma (fibroblasts, endothelial cells, infiltrating immune cells, …) During aging, the probability that a senescent fibroblast and a premalignant (epithelial) cell (due to low grade DNA damage) come to lie in each other's microenvironment, increases. It has been shown that these premalignant cells then lose differentiated properties, become invasive and undergo full malignant transformation[3,31-33]. Several preclinical experiments confirm this hypothesis: malignant epithelial cells that were injected together with senescent fibroblasts into xenografts, showed much more rapid growth compared to malignant epithelial cells alone[32,34]. And even in non-malignant breast epithelial cells, senescent fibroblasts have been shown to disrupt the epithelial alveolar morphogenesis, the functional differentiation and the branching morphogenesis[31].

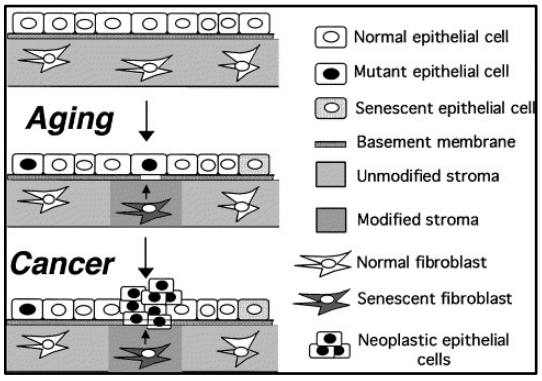

Figure 5: Taken from Krtolica et al [3], with permission from Elsevier.
A model for synergy between mutations and cellular senescence in the occurrence of age-related cancer.

The inflammatory microenvironment of the aging prostate has been suggested to be stimulatory on the proliferation of both epithelial cells and fibroblasts[35], and older stromal prostate cells, when

cultured in vitro, were shown to exhibit stimulating effects on tumor formation by epithelial cell lines (benign and cancerous)[36].

The SASP is composed of several degrading enzymes and cytokines that modify the stroma such that is resembles an active stroma[3,33,37,38]. One of the most important components of the SASP has been suggested to be matrix metalloproteinase 3 (MMP3)[31]. This metalloproteinase has also been shown to promote mammary carcinogenesis[39]. The SASP of fibroblasts can be further composed of inflammatory cytokines and immune-modulatory chemokines (e.g. IL-6, IL-7, IL-8, MCP-2, MIP-3a), shed surface molecules (e.g. ICAMs, uPAR, TNFreceptors), growth- and survival factors (e.g. GRO, HGF, IGFBP). It is not a fixed phenotype, but a fluctuating profile with broad overlap between cell types and growth conditions[37,40,41].

d. The autophagy to senescence transition

An additive mechanism that has been proposed to explain the tumor promoting effects of a senescent microenvironment is "the autophagic tumor stroma model of cancer"[42-45]. This model states that fibroblasts, in transition to a senescent state, activate the autophagy process. It is therefore also called the Autophagy to Senescence transition (AST). The fibroblasts thereby shift towards an aerobic glycolysis-metabolism, creating high-energy mitochondrial fuels that feed the epithelial cancer cells. The discovery of the AST was driven by the finding that tumoral cells were capable to influence surrounding fibroblasts to undergo AST, by secreting hydrogen peroxide, which induced oxidative stress in the fibroblasts and resulted in activation of autophagy. This process was named "the Reverse Warburg Effect". Fibroblasts displaying a constitutively activated autophagy program, turned out to show many morphological characteristics of senescence, as well as induction of p21(WAF1/CIP1). Moreover, they were shown to promote tumor growth and metastasis, when co-injected with human breast cancer cells[42] which led to the hypothesis that AST is one of the mechanisms by which senescent stromal cells create a 'fertile soil' for the occurrence and progression of cancer.
Typical genes associated with autophagy are BNIP3, CTSB or ATG16L1[42,46-48]. Also, loss of CAV-1 expression has been shown to be a biomarker for autophagy in stromal cells, and has been shown to correlate with a lethal tumor microenvironment[49].

2. Breast cancer in older patients
    a. Rising incidence and worse outcome

Epidemiological studies expect the number of individuals over the age of 65 years to double by the year 2030[50]. Centenarians will be the fastest-growing subpopulation. In this population the association between cancer and aging is of particular interest. Approximately 60% of cancer incidence and 70% of cancer-related mortality occurs in individuals aged older than 65 years[50]. Breast cancer is a frequent disease in our community. In Europe, incidence for women 70 years or older diagnosed between 2000–04 varied from 100 to 350 per 100000 per year[51]. The most recent publically available data of the Belgian National Cancer Registry, show 10531 new breast cancer diagnoses in 2012, from which 3354 diagnoses are made in women of 70 years and older. The curves representing the age-standardized (using the World Standard Population) incidence of breast cancer, per age group, are shown below in figure 6..

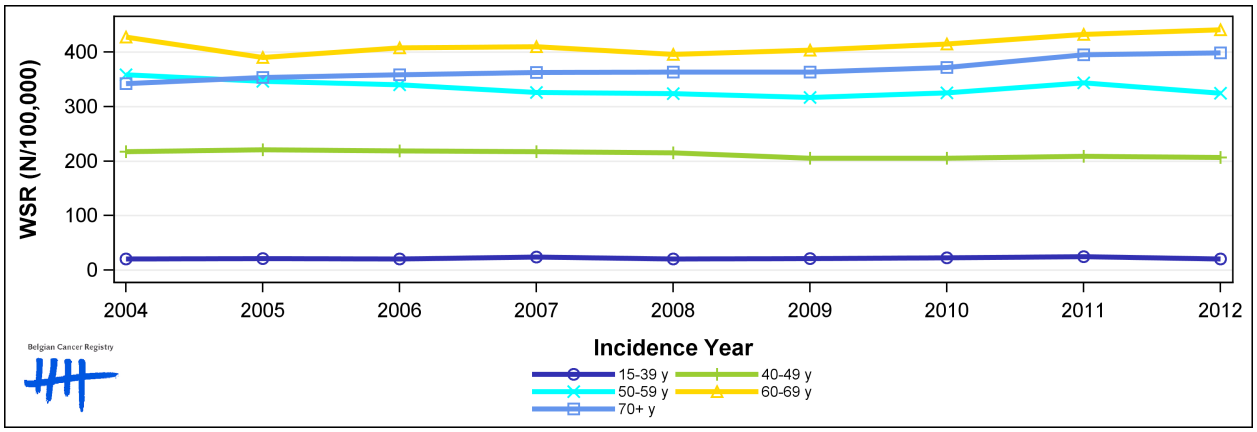

Figure 6: Age-standardized (using the World Standard Population) incidence of breast cancer in Belgium, by age group and incidence year. Source: Incidence Fact Sheets, Stichting Kankerregister, Incidentiejaar 2012, Brussel 2015

Breast cancer does not present as a uniform disease. Breast cancers differ in microscopic appearance and biologic behavior. The invasive breast carcinomas consist of several histologic subtypes, from which infiltrating ductal carcinoma represents the most frequent subtype. Other subtypes are invasive lobular, mixed ductal lobular, mucinous (colloid), tubular, medullary or papillary carcinomas. In most studies, the prevalence of tumors with more indolent features is higher in older compared with younger women. There are higher rates of hormone receptor expression[52-55] (85 versus 70 percent in women ≥65 versus <50 years, respectively), lower rates of HER2 overexpression[56,57], and a higher proportion of low-risk tumor histologies. As in younger women, infiltrating ductal carcinoma is the most common histologic type of breast cancer in the elderly population, however more indolent breast cancers (eg, mucinous and papillary carcinomas) are encountered more often with advancing age[52,58,59]. The incidence of hormone sensitive breast tumors differs mostly between very young patients and older patients, but there is less variation

between age groups among postmenopausal women[60,61]. A more recent way of classifying breast carcinomas is based on their gene expression profiles giving rise to different molecular subtypes. Luminal A and B, Her2-enriched, and ER negative (claudin-low and basal-like) subtypes exist. Luminal tumors are more often found in elderly patients, while Her2-enriched, basal-like and unclassified subtypes were more often found in young patients[62,63].

b. Lack of evidence based treatment choices

Older patients are often diagnosed in a more advanced stage of the disease[64], fear and hesitation to search medical attention probably explains this, at least in part. Most importantly, older patients are often withheld therapy, which worsens their prognosis[65]. This could be due because physicians fear to cause excessive toxicity by implementing similar treatments as is younger counterparts, or because they consider the treatment as futile. Whatever the reason may be, it shows the need to expand the evidence supporting treatment (or no treatment) decisions in this population. Only very few evidence-based guidelines exist in older patient groups, because in the past they were typically excluded from clinical trials.
With the current progress that is made in oncology research and therapy development, and the expected increase in cancer in a growing older population, it is of utmost importance to be able to consider all treatment possibilities for these patients, and not just deny them therapy based on assumptions.

3. The concept of frailty, biological age, and the heterogeneity of the patient population

The difficulty in studying a 'geriatric population' with cancer, is it's heterogeneity and multidimensionality. The definition of a geriatric patient is not only based on advanced age, but on a combination of age, medical problems or diseases, and limitations in several functional domains of the organism. Taking care of this population requires a holistic approach taking into account medical, functional and psychosocial aspects that can vary between patients. Cancer can arise in fit older patients as well as in very frail people, representing both ends of a spectrum. In other words, patients can be of the same calendar age, but their underlying biological age might be different.

The syndrome of frailty has been extensively studied in geriatrics. It is not a simple synonym of disability or comorbidity, it represents a distinct biological process. Fried's description of the frailty phenotype[66] is often used: it is based on five pre-defined physical frailty criteria: unintentional

weight loss, self reported exhaustion, weakness/grip strength, slow walking speed and low physical activity.

In extremely frail patients, a new diagnose of (breast) cancer often results in the decision to treat at most with endocrine therapy, which can be expected not to induce severe toxicity. These patients do often not present with the most difficult treatment decisions. More difficult are situations where the level of frailty of the patient is intermediate or where the patient is seemingly fit but very old. Especially in breast cancer, where the therapeutic possibilities are often various, and the cancer-specific prognosis on average good. This is the reason why we chose this type of cancer as the model for our research.

Aging of the entire body is reflected at different levels. First of all, it can be associated with clinical signs of aging, such as a decline in cognitive and physical performance.
Secondly, aging can be measured in different biological systems by determining biological markers of aging. The blood is the most easily accessible organ to measure biological markers of aging (Fig 7).

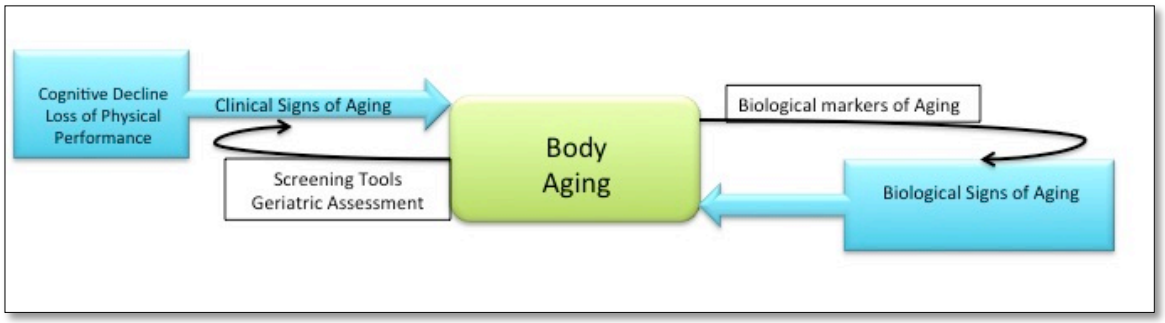

Figure 7: Body aging: the aging process can be measured by clinical scoring systems or through measurement of biological markers

a. <u>The rising use of a (Comprehensive) Geriatric Assessment</u>

Oncologists have set up a society, specifically focusing on geriatric patients in oncology, with the purpose of developing more scientific evidence-based guidelines for treatment decisions in this heterogeneous population: SIOG, the International Society of Geriatric Oncology. SIOG strongly suggests to perform a geriatric assessment in all older patients diagnosed with cancer[67-69].

A Comprehensive Geriatric Assessment (CGA), also called Geriatric Assessment (GA), is defined as a 'multidimensional, interdisciplinary diagnostic process focusing on determining an older person's medical, psychosocial and functional capability in order to develop a coordinated and integrated plan for treatment and long-term follow-up'[70]. The core components of a GA are social support, functional status, fatigue, comorbidity, cognition, mental health status, nutrition, and geriatric syndromes (e.g., dementia, delirium, falls, incontinence, osteoporosis or spontaneous fractures, neglect or abuse, failure to thrive, constipation, polypharmacy, pressure ulcers, and sarcopenia). In order to assess these domains, several standardized questionnaires are being used. An example of a comprehensive geriatric assessment that can be used (used by us in the Elderly Biomarker Study, cfr chapter 3), is shown in appendix 1.
Ideally, all older cancer patients should be evaluated by CGA followed by interventions. But because geriatric assessment is a time-consuming activity, several screening tools have been developed to quickly assess all patients, and select patients that need a more in depth evaluation[71]. A screening tool is a brief assessment. They should be simple and quick. The purpose of this two-step approach is to identify in a time-efficient way, which patients are in need of guided multidisciplinary interventions[72]. High sensitivity and negative predictive value are the most important characteristics. In addition, a high specificity is of interest in order to limit the number of unnecessary CGA's. One of these screening tools, the G8, is included in appendix 1. Other examples are the VES-13 (Vulnerable Elderly Survey-13), TRST (Triage Risk Screening Tool), GFI (Groningen Frailty Indicator), TUG (Timed Up and Go). Many others exist. In a systematic review, G8 was described to be the most robust, but no specific screening tool can be recommended because performance of these tests can be situation-dependent[71].

b. Summarizing the CGA – Balducci

The geriatric assessment serves in the first place as an in depth assessment[73], to learn more about the sometimes hidden functional problems of patients. The result of the geriatric assessment should be taken into account when making oncological decisions. However, the multidimensional result makes interpretation of the 'level of frailty' that results from it, especially for oncologists with less geriatric experience, difficult. A certain quantification of the frailty level would make the implementation of the geriatric assessment into clinical decision-making easier. In medical oncology, we look for a definition of frailty capable to identify older subjects with a critical reduction in functional reserve, that makes them unsuitable for standard forms of cancer treatment, and mandates individualized treatment plans.

In general medicine, there are two main models known to define frailty: The Fried Criteria and the Cumulative Deficit Model. These models offer criteria and/or tests to classify patients into categories of fitness. They have however not been validated in an oncological population and do not always hold an answer for treatment decisions. For that reason, L. Balducci suggested a classification based on the results of geriatric assessment, which specifically classifies onco-geriatric patients into the categories FIT – VULNERABLE – FRAIL[74,75]. According to his publications, fit patients are patients that are functionally independent and without comorbidity, and who are candidates for any form of standard cancer treatment (with the possible exception of bone marrow transplant). Frail patients are only candidate for best supportive care, and vulnerable patients represent the category in between. They may benefit from some sort of pharmacological intervention but with personalized adaptations.

Fit is hereby defined as age <85, no limitations in ADL or iADL, no (or mild) comorbidities and no geriatric syndromes. Frail is defined as age ≥ 85 years and/or dependence at ≥ 1 item of the ADL and/or ≥ 3 comorbid conditions and/or ≥1 geriatric syndrome (dementia, falls, delirium, depression, incontinence, osteoporosis, neglect and abuse, failure to thrive). Vulnerable patients are the ones in between, showing dependency at 1 or more iADL items, and/or with 1 or 2 severe comorbidities.

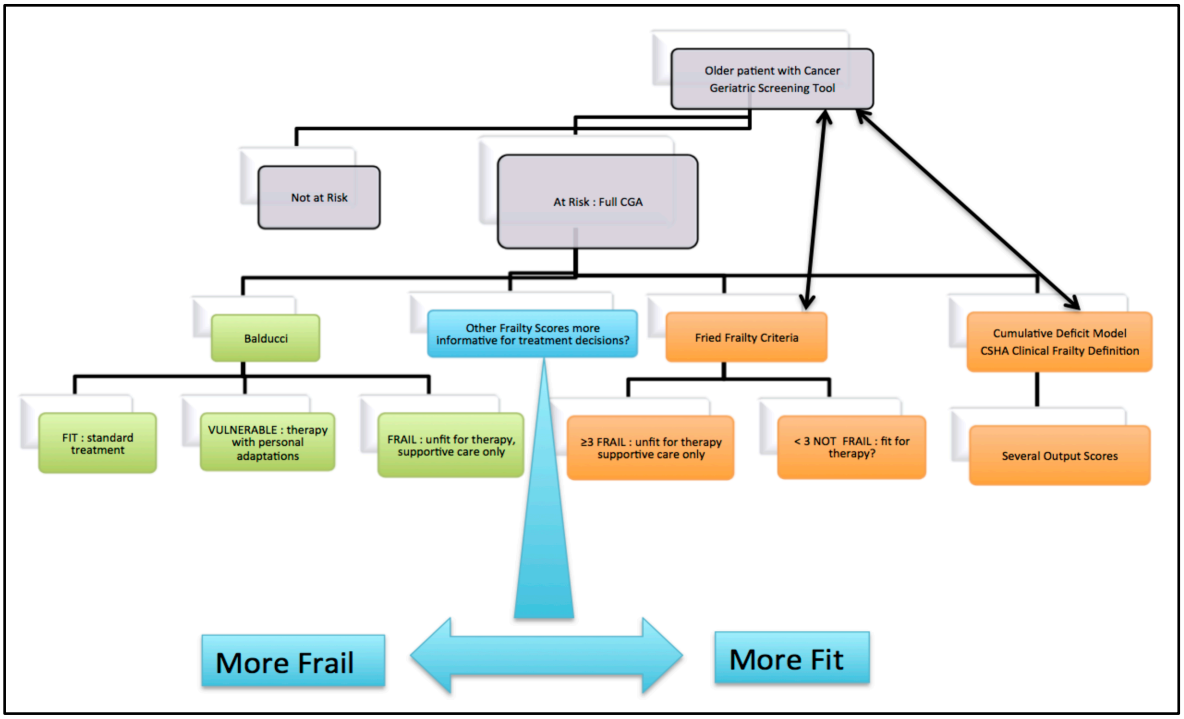

Figure 8: The landscape of frailty evaluation. Green tests have been developed in oncological patients – yellow tests are being extrapolated from the general geriatric population to oncological patients

The classification of Balducci represents an important attempt to make the geriatric assessment part of the oncological decision process. However, it represents a quite rough way of categorizing patients. Moreover, classifying patients above 85 years old, or patients with occasional incontinence

by default, as frail, holds again the risk to deny a useful treatment to the patients that could be fit enough to receive it.

c. Biological markers of aging

Besides the clinical measures of frailty, which reflect the clinically measurable consequences of the aging process, a lot of interesting research has been performed on the biological markers that are thought to reflect the underlying physiological process of biological aging and that might be useful in assessing a patients ability to tolerate cancer therapy.

Telomeres

A crucial role has been attributed to telomeres, in cells and tissues subjected to replicative aging. Telomeres are DNA–protein complexes of repetitive DNA sequences and telomere binding proteins[76]. They cap chromosomal ends in order to preserve chromosomal stability[77]. They are incompletely replicated in somatic cells and shorten with each cellular division until reaching a critical value. At that point, genomic instability occurs, and senescence mechanisms are activated[78]. Only germ cells and stem cells (and often also cancer cells) express telomerase, a reverse transcriptase that can re-elongate shortening telomeres again[79]. In somatic cells, there is no or little telomerase activity[80]. As a consequence, the number of cell divisions is limited (this is called the 'mitotic clock' of a cell). Therefore, leukocyte telomere length (LTL) can serve as a marker of a cell's replicative "age"[81], and, in extension, can mirror a person's biological age[82]. In a population-based cohort study with 3075 healthy, well-functioning men and women aged 70-79 years[83] leukocyte telomere length, although not associated with overall survival (HR 1.0; 95% CI 0.9-1.1) or death from any specific underlying cause including cancer, was positively associated with more years of healthy living. According to the authors, these findings suggest that although such crude assays of average telomere length may not yield strong biomarkers of survival in older individuals, they may be informative for healthy aging. LTL also correlates with several aging-related syndromes[84] such as cardiovascular diseases[85], heart failure[86], osteoporosis[87] and obesity[88].
It should be acknowledged that telomere length measurement techniques have intrinsic technical limitations, and that large inter-individual differences exist of which the biological meaning is not well known. Measurement of telomere length can be performed in different ways (mean telomere length by RT-qPCR or TRF, shortest telomere by FISH) each with their own advantages and disadvantages[89]. Although telomere biology is extremely interesting, the (prognostic/predictive) value of telomere length in an individual patient has not yet been established. This might be because

most often, average telomere length of all chromosomes is measured, whereas the shortest telomere length may be the crucial trigger for eg. replicative senescence, regardless of the overall value.

Expression of $p16^{INK4A}$ in lymphocytes

Lymphocyte senescence, and thus aging of the immune system, is reflected by increased mRNA expression of the cell cycle regulator $p16^{INK4a}$ [6]. As described before, the $p16^{INK4a}$ gene acts as an important regulator of senescence. It works as inhibitor of cyclin-dependent kinase 4/6. In healthy humans, $p16^{INK4a}$ expression in peripheral blood T lymphocytes increases markedly with physical inactivity and exposure to mutagens such as tobacco[90]. Also, T lymphocyte expression of $p16^{INK4a}$ increases exponentially with chronological age, with an average 10-fold increase between the ages of 20 and 80[91]. Although expression of $p16^{INK4a}$ in T-lymphocytes seems a very promising aging biomarker, the use of it is hampered by some technical challenges. It has to be determined on mRNA extracted from T-lymphocytes, which is not readily available in routine blood samples. As a solution, mRNA from total blood leukocytes can be used, although the correlation with age showed much less strong by using that approach.

Inflammatory cytokines and immune regulating chemokines

A role for inflammation in the process of aging and age-related disease has been clearly established in several large epidemiological studies of older adults. Although acute inflammatory responses are closely regulated in the elderly, a low-level elevation of inflammatory markers is commonly observed and is associated with several chronic conditions of aging such as physical and cognitive decline, cardiovascular diseases and diabetes, or cancer[92]. While antigen-directed/adaptive immune responses usually decline with aging, general/non-specific inflammation seem often to increase in the older patients, a phenomenon termed inflamm-aging[93]. Several reports have described an increase in pro-inflammatory cytokines (mainly interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α)), chemokines (Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), and Monocyte Chemotactic Protein 1 (MCP-1)) and C-reactive protein (CRP), with increasing age even in healthy older people, while at the same time there is a decrease in anti-inflammatory mediators such as interleukin-10 (IL-10)[92,94,95].

The etiology of this rise in inflammatory status has not been entirely clarified. It is thought to be a result of several contributing factors such as cumulative oxidative damage that promotes inflammatory responses, declining levels of sex hormones after menopause and andropause[92], increasing visceral adiposity, and chronic immune stimulation by persistent irritants especially latent viral infections, most particularly Cytomegalovirus[96]. It is not clear to what extent a systemic

repercussion of the local microenvironment changes induced by senescent cells contributes to this inflamm-aging, but a relationship has been suggested[97].

In community-dwelling elderly there is a clear association between IL-6 levels and functional disability[98] and a "frailty" phenotype[99]. Interestingly, peripheral cytokine levels are often elevated prior to cognitive decline[100], dementia[101] and loss of physical performance[102]. Furthermore, markers of inflammation are considered as a predictive tool for mortality[103,104].

However, an important issue that limits the clinical use of inflammatory factors as potential aging biomarkers is the fact that their blood levels are dependent on inflammatory reactions caused by underlying health conditions. The timing of testing may therefore represent an important confounding factor, e.g. assessing inflammatory factors in patients post-operatively after cancer resection to judge longevity/fitness for adjuvant chemotherapy, might result in misleading conclusions because recent surgery itself most likely increases IL-6/CRP levels. Moreover, there is no clarity on the contributing factor of neoplasia in the rising inflammatory parameters, which might also be very variable according to the type of malignancy involved.

Insulin/insulin-like growth factor 1 (IGF-1) metabolic pathway

Numerous studies have shown that aging is hormonally influenced by an evolutionarily conserved insulin/IGF-1 signaling (IIS) pathway[105]. Caloric restriction has been proven to be successful in prolonging life span at least in rodents[106]. Levels of insulin-like growth factor 1 (IGF-1) have been shown to decline with advancing age[107], but more interestingly, IGF-1 has also been mentioned as predictive for functional status. Associations with muscle strength, slow walking speed, and self-reported difficulty with mobility tasks have been shown, as well as increased mortality with lower IGF-1[108,109].

Other potential biomarkers

Several other potential biomarkers reflecting the aging process have been described. For example markers of telomere dysfunction[110]: cathelicidin-related antimicrobial peptide or CRAMP, stathmin, Elongation Factor - 1α or EF-1α, and chitinase-3-like protein. These factors are secreted by bone marrow cells with short, dysfunctional telomeres, but not from cells with long telomere reserves. They can be detected in the serum/plasma of patients, and were found to be higher in older people, certainly in patients with comorbidities. Furthermore, changes in immune cell subsets and CMV serology results, can be compiled into an immune risk profile (IRP) comprising an inverted

CD4:CD8 ratio, accumulation of CD8+CD28- late-differentiated T cells, poor proliferative capacity, few B cells and CMV-seropositivity. This IRP has been shown to be associated with significantly increased 2-, 4- and 6-year mortality in very old people[111,112]

All these biological markers have been described in association with the aging process, but none of them can be considered as the best biomarker of aging, and the value of these markers at the individual level has not yet been established. The most robust ones are leukocyte telomere length and IL-6, but probably all of them reflect a small part of the intrinsic aging process.

4. Chemotherapy in Older patients

Decisions on chemotherapy in older patients are difficult: oncologists should try not to deny therapy to patients fit enough to receive it, but they fear excessive and sometimes irreversible toxicity which is clearly more frequent in older patients compared to younger patients. Toxicity especially in older patients can present on the short-term: the well-known acute chemotherapy toxicity, but also on the longer term reflected by a progressive increase in frailty level, which could be related to an acceleration in the aging process caused by the treatment. For the short-term toxicity some parameters of the geriatric assessment are known to be predictive, these are summarized in the two scores that we further elaborate on in the next paragraph. This is the reason why a thorough assessment of a patient's frailty level is advised before taking decisions on chemotherapy treatments. The value of aging biomarkers in refining these scores has never been tested.
A few studies have made an attempt to investigate the effect of chemotherapy on the aging process by measuring some aging biomarkers, thereby trying to provide an explanation for longer-term toxicity. These studies are highlighted in the second paragraph. However, the findings are not uniform, and neither one of these studies has included clinical parameters to document if the changes in biomarkers do actually correspond to clinical changes.

a. Prediction of short-term chemotherapy toxicity: CRASH score and Hurria Score

Two studies have investigated the predictive value of geriatric assessment parameters for short-term chemotherapy toxicity
The CRASH score developed by M. Extermann[113] et al, uses simple parameters from routine clinic and geriatric assessment (diastolic blood pressure, instrumental activities of daily living (iADL), Lactate Dehydrogenase (LDH), ECOG performance status, Mini Mental State Examination (MMSE) and Mini Nutritional Assessment (MNA)) in combination with an assumption about the

intrinsic chemotherapy risk (called "Chemotox value"). This score stratifies patients into four risk categories (low, medium-low, medium-high and high) for developing hematological or non-hematological toxicity after treatment with chemotherapy. Although this score, published in 2012, seems quite easily applicable into clinical practice, it has not gained a lot of popularity. One of the explanations might be that it involves an unknown chemotherapy-toxicity score (the Chemotox value) that oncologists are not familiar with. It is a score developed by the authors themselves, based on toxicity data of previous published trials that reflects the risk of toxicity for a given regimen, in a 'standard' patient.

The predictive model developed by A. Hurria[114] et al is more complex. It takes into account a lot of parameters (age, type of cancer, planned chemotherapy dosing, mono- versus polychemotherapy, hemoglobin (with different cut-offs for male and female), creatinine clearance, hearing impairment, number of falls in the last 6 months, help needed with taking medications, ability to walk 1 block, decreased social activities due to health (physical or emotional) problems). Based on these parameters, a total score can be calculated, ranging from 0 to 19, trichotomized into three categories with a different risk of treatment toxicity. This score as well, has not found entrance to the broad clinical practice, probably because of its complexity.

Never has there been any study investigating the value of biological aging markers in predicting short-term chemotherapy toxicity.

b. <u>Prediction of long-term chemotherapy toxicity: influence of chemotherapy on the aging process</u>

Chemotherapy may influence the aging process via a variety of different mechanisms. Firstly, anticancer agents can induce cellular senescence through DNA damage[115], either directly or indirectly via generation of free radical intermediates and inhibition of DNA repair enzymes. Secondly, chemotherapy may specifically accelerate telomere attrition in leukocytes, most likely due to direct telomere damage or possibly by inhibition of the enzyme telomerase[116]. Repeated cycles of intense haematological repopulation during chemotherapy may shorten telomeres more rapidly if telomerase is not compensating for endochromosomal DNA loss[117-119]. Such effects of anticancer drugs on the replicative capacity of blood cells may be more pronounced in older compared to younger patients[120]. Finally, neuroendocrine and immune functions can also be affected by chemotherapy and by corticosteroids that are often incorporated in chemotherapeutic regimens[121]. Chemotherapy might thus be expected to accelerate aging[122] and this might be responsible for increasing frailty on the longer term after chemotherapy. There have already been some attempts to study this topic.

In vitro work on telomere length shortening in mesenchymal stem cells after chemotherapy treatment suggests a shortening effect of this treatment[123]. In 2002 Lee et al investigated leukocyte telomere length shortening in Non-Hodgkin lymphoma patients[124]. They followed up on 5 patients that received chemotherapy and measured leukocyte telomere length before and after the therapy. They found a decrease in mean telomere length when comparing the values before and after chemotherapy. In 2006, Unryn et al published a study on telomere shortening during chemoradiotherapy for Head and Neck Cancer[120]. Patients underwent blood sampling for measurement of leukocyte telomere length at diagnosis, during and after chemoradiotherapy. These blood samples showed a faster decrease in telomere length in patients receiving chemotherapy compared to historical controls (belonging to another research project). A third study investigated telomere dynamics in BRCA mutated breast cancer patients compared to other familial, or sporadic breast cancers. In the group of sporadic breast cancers they only found a transient decrease in telomere length that recovered to normal age-expected values after 2 years[125]. So they did not entirely replicate the findings of the two previous studies. Finally, in 2014 a study was published by Sanoff et al,[126] not only investigating telomere length, but also several other aging markers before, during and after chemotherapy for breast cancer: $p16^{INK4a}$ expression and senescence-associated cytokines VEGFA, IL-6, IL-7, IL-8 and MCP-1. They found and increase in expression of $p16^{INK4a,}$ which might be compatible with an accelerated aging process, but did not find a decrease in leukocyte telomere length, thus refuting the findings of previous studies. VEGFA and MCP-1 were found increased after the chemotherapy treatment whereas the rest of the cytokines remained stable.

Taken together, there are only a few studies investigating the effect of chemotherapy on the biological aging process, and these are of variable quality, as we will further elaborate on in the discussion of this thesis manuscript. Some of them suggest an increased rate of biological aging due to chemotherapy. But their findings are inconsistent with each other, and this together with the methodological shortcomings of these studies, makes the issue unresolved in our opinion.
This is a difficult topic to study, as there is no readily available test to measure with certainty the biological age of a person. Most of the studies focused mainly on leukocyte telomere length, Sanoff et al also measured $p16^{INK4A}$ expression in T-lymphocytes, next to a few age-related cytokines. None of them included geriatric assessment on top of the biomarker results.

The main objective of this doctoral research was to expand the scientific knowledge on the complex interface between cancer and biological aging. We chose the platform of breast cancer to do so, because of the high incidence of this disease, which will make our findings relevant for a broad population, as well as the different therapeutic options, making the prognosis of the disease fairly good even in older patients, provided that it is correctly treated. Moreover, most of the research work that we based our assumptions on has also been performed in breast cancer.

As a first objective, we were interested to know if stromal characteristics would be different between breast cancers occurring in young patients, and breast cancers occurring in older patients. Based on the description of the SASP (senescence associated secretory profile) and AST (autophagy to senescence transition), and the hypothesis that senescent cells accumulate in the body with aging, tumors arising in older patients would be expected to display a stromal compartment with different characteristics. Senescence in the surrounding stroma is expected to result in a pro-tumorigenic microenvironment with stimulation of proliferation, migration/invasion, and dedifferentiation. But never had this been shown in spontaneous occurring breast cancers. Most experiments that deal with the interaction between senescence and cancer start from fibroblast cultures, where senescence has been artificially induced. The altered phenotype and functional activity of this type of fibroblasts are then shown to influence the behaviour and growth speed of (pre)malignant cells, in cell cultures as well as in xenografts. As a consequence of this approach, an overload of senescent or presenescent fibroblasts is present in these experiments. We don't know if this reflects accurately the situation in spontaneous cancers. Accumulation of senescent cells with age has mostly been studied in fibroblasts localized in the skin[16], but data on the frequency of senescent fibroblasts in the older breast are lacking. Moreover, controversy exists on whether we can extrapolate findings on in vitro senescence, to the situation in vivo.

A second reason why we wanted to challenge the preclinical findings on this topic is the fact that they are difficult to reconcile with the experience in clinical practice. Cancers are more frequent at older age, but little evidence can be found for a more aggressive behavior of these tumor cells: breast cancers in older patients have on the contrary been shown to grow slower[127] (in general), and to behave less aggressively, even when adjusting for different histological tumor characteristics[128].

We decided to study the biological aging process in the older human breast cancer microenvironment, and look for in vivo confirmation of key concepts such as senescence, DNA

Damage Response, SASP, and AST. For this purpose, we selected two groups of breast cancer patients, belonging to a young respectively old age category, and used Laser Capture Microdissection to separate stromal fields from the cancer cells.

Next, we wanted to broaden our scope, and investigate the biological aging process in the rest of the host organism. We were interested to know how biological age can be accurately assessed in cancer patients, and if quantification of this aging process would allow us to refine treatment decisions in older breast cancer patients.

As described in the introduction, a jungle of clinical and biological parameters exist that can reflect to a more or lesser extent the frailty level in older patients. Clinical evaluations are time consuming, the multidimensional result of these assessments might not always be easy to interpret for oncologists with less experience in the geriatric field, and not easy to implement into oncological decision making. Biological markers on the other hand, are highly variable, influenced by several confounding factors and the measurements are sometimes associated with technical challenges. In the field of gerontology, the association of aging and frailty with these markers has intensively been studied. Nevertheless, no single marker can yet be considered a validated biomarker of aging or frailty. Moreover, simple extrapolation of findings from a population without cancer, to the oncological setting has been debated: cancer lesions and the potential physiological changes that are caused by their presence might influence levels of these biomarkers.

In a second chapter of this thesis, we therefore present a retrospective study, where we have been investigating the value of known biological markers, suggested to reflect aging and frailty, in an older breast cancer population. We also developed an alternative way of summarizing the result on GA, in order to obtain a single clinical score reflecting the 'frailty level'. This LOFS (Leuven Oncogeriatric Frailty Score) could be easier to use in oncological decision-making compared to the full GA result, but on the other hand more sensitive for subtle changes in frailty than the currently known Balducci categories.

In the third chapter of this thesis, we deal with a very important question for clinical practice: does chemotherapy treatment influence the biological aging process? We wanted to prospectively follow up on signs of aging (clinical by geriatric assessment, as well as biological by measuring biomarkers) in older patients receiving either or not chemotherapy for early breast cancer (without metastases). If chemotherapy would show to accelerate aging, this would be an important finding to take into account while deciding on indications for chemotherapy treatments. On the other hand,

absence of an aging-inducing effect might convince treating oncologists not to deny chemotherapy to fit patients that would derive significant benefit from it. We performed a prospective observational study following older breast cancer patients during and after chemotherapy, as well as a group of breast cancer patients not receiving chemotherapy. Besides the question if chemotherapy would accelerate biological aging, we investigated if frailty levels at diagnosis (assessed by geriatric assessment and biological markers) could be used to predict short-term chemotherapy toxicity or unplanned readmissions in hospital.

This paper is in preparation for submission

Title

"The footprint of the aging stroma in older breast cancer patients."

Authors

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(*) shared first authorship, these authors contributed equally to the research project

(**) shared last authorship

ABSTRACT

Introduction: Tumoral masses are not only composed of malignant cells, but also enclose a more or less ample stromal micromilieu, which has been shown to influence the cancer cell behaviour. As aging induces accumulation of senescent cells in the body, this micromilieu is thought to be different in cancers occurring in old patients compared to the younger counterparts. More specifically, senescence-related fibroblastic features, such as the Senescence Associated Secretory Profile (SASP) and the induction of Autophagy, are suspected to stimulate tumor growth and progression.

Materials and Methods: We compared gene expression profiles in stromal fields of breast carcinomas by performing laser capture microdissection of the cancer-associated stroma from 8 old (≥80 years at diagnosis) and 9 young (< 45 years at diagnosis) triple negative breast cancer patients. Gene expression data were obtained by microarray analysis (Affymetrix). Differential gene expression and Gene Set Enrichment Analysis (GSEA) were performed.

Results: Differential gene expression analysis showed higher growth-, dedifferentiation- and migration- promoting gene expression in the stromal samples of older vs younger patients. GSEA confirmed the presence of a SASP, as well as the presence of Autophagy in the the stroma of older patients.

Conclusion: We provide the first evidence in humans that older age at diagnosis is associated with a different stromal micromilieu in breast cancers. The SASP and the presence of Autophagy appear to be important age-induced stromal features.

Introduction

Oncology research over the past years has been focussing in the first place on tumor cell characteristics. However, tumoral masses are not exclusively composed of malignant cells, but also comprise a stromal component containing endothelial cells, (myo)fibroblasts, smooth muscle cells, adipocytes and inflammatory cells. Research on the stromal component of tumor masses has shown that stromal characteristics are correlated with disease outcome and behaviour[1-10] in several malignancies. The stroma seems to play a very important role in tumor initiation, progression and metastatic spread[11,12]. The fibroblasts contained in this stromal compartment show a specific phenotype and are called 'carcinoma-associated fibroblasts' or CAFs[13]. As cellular senescence progressively occurs throughout lifetime in fibroblasts of various origins[14], it seems plausible that the characteristics of the stromal compartment of breast cancers would differ between young and older patients.

The incidence of breast cancer, the most frequent tumor occurring in women, increases with age[15,16]. Whereas breast cancer at young age usually reflects either a genetic defect or the impact of early life transforming effects on an immature breast epithelium, cancer in older patients is thought to arise from life-long exposure to harmful stimuli, such as DNA damaging agents, oxidative stress factors and telomeric loss. In addition, the microenvironmental changes caused by senescent cells might also be an important harmful trigger.

Senescence in general is a protection mechanism that shuts down damaged cells[17]. Senescent cells are forced into a state of irreversible growth arrest[18,19] and exhibit a specific phenotype characterized by enlarged size, flattened morphology, senescence-associated β-galactosidase activity, reorganization of chromatin into foci of heterochromatin and resistance to apoptosis[20]. They also acquire the so-called Senescence Associated Secretory Profile (SASP)[21,22], maintaining the growth arrest and recruiting immune cells towards the damaged cells, in order to eradicate them. However, the SASP also seems to have a detrimental influence on nearby cells. Epithelial cells neighboured by senescent fibroblasts lose differentiated properties, become invasive and undergo full malignant transformation[20,23-25]. In this process, a major role has been attributed to the matrix metalloproteinase 3 (MMP3)[23] together with other components of the SASP[25,26], such as inflammatory cytokines and chemokines. This concept of senescence as useful cancer-protective mechanism in younger life but detrimental cancer-promoting mechanism in later life has been repeatedly described as an example of "antagonistic pleiotropy[27,28]" in cellular or animal models[23,24,29-32].

An additional mechanism that has been proposed to explain the tumor-promoting effects of a senescent micro-environment is the "the autophagic tumor stroma model of cancer"[33-36]. This model states that fibroblasts, in transition to a senescent state, activate the autophagy process. During this so-called Autophagy to Senescence Transition (AST), the cells shift towards an aerobic glycolysis-metabolism, creating high-energy mitochondrial fuels that feed the nearby epithelial cancer cells. Autophagic fibroblasts were shown to have tumor- and metastasis-promoting activity[34]. The discovery of this concept was preceded by the finding that tumoral cells can induce AST in surrounding fibroblasts, by secreting hydrogen peroxide that causes oxidative stress and activation of autophagy in the fibroblasts. This process was named "the Reverse Warburg Effect", (as opposed to the original idea, called "the Warburg effect", that aerobic glycolysis takes place in epithelial cancer cells). Fibroblasts displaying a constitutively activated autophagy program turned out to show many morphological characteristics of senescence, including induction of p21(WAF1/CIP1), which led to the hypothesis that AST is one of the mechanisms by which senescent stromal cells create a 'fertile soil' for the initiation and progression of cancer.

Despite this knowledge, stromal differences with increasing patient age have so far never been investigated in vivo and little clinical evidence can be found for a more aggressive behavior of tumor cells growing in a context of 'older' stroma. On the contrary, breast cancers in older patients have in general been shown to grow more slowly and to behave less aggressively, even when adjusting for different histological tumor characteristics[37].
On these premises, we decided to compare the gene expression profile of tumor-adjacent stroma in older versus younger breast cancer patients paired for other clinico-pathologic parameters.

Materials and Methods

Patient Selection and clinical specimens

This study was approved by the Ethical Committee of the University Hospitals Leuven (Leuven, Belgium), in accordance with the International Conference on Harmonization Guidelines on Good Clinical Practice.

Candidate patients were selected using the following criteria: 1) < 45 years or ≥ than 80 years old; 2) no neo-adjuvant chemotherapy treatment nor hormonal treatment before surgery; 3) surgery for early triple negative breast cancer (estrogen and progesterone <1%, and HER2 <2 by immunohistochemistry or FISH negative) and fresh-frozen resection specimens available (stored at -80°C at the Pathology Department of the University Hospitals Leuven); 4) no chronic inflammatory diseases to exclude confounding variables.

From candidate patients, 1 section of the frozen tumor material was obtained for haematoxylin and eosin (H&E) staining. H&E sections were evaluated for tumoral and stromal content to localize the best areas for stromal microdissection (see Figure 1 as an example). Only tissue blocks consisting of invasive tumor with sufficient carcinoma-associated stromal fields to allow laser capture microdissection were selected. Tumors with >20% of infiltrating lymphocytes were excluded to prevent biases in the gene expression analyses.

On the bases of the above criteria, 17 female breast cancer patients (9 young patients <45 years at diagnosis and 8 old patients ≥ 80 years at diagnosis) were eventually included in the study.

Staining Procedures and Laser Capture Microdissection(LCM)

Preparation of the Tissue Slides

For the selected patients, 10 frozen sections of 10 μm thickness were mounted onto specific membrane slides (Steel frames with PET membrane from Leica, cat # 11505151) and were kept at -80°C until the staining and dissection procedure was started. All tissue slides underwent LCM within 7 days after preparation.

Staining

Prior to LCM, tumor slides were stained with Cresyl Violet following a procedure optimized for maximizing RNA yield. Briefly, tumor slides were taken from -80°C and were fixed into a 95% ethanol solution for 30 seconds. Next, they were transferred to ethanol solutions with progressively decreasing concentrations (75%, 50%) for 30 seconds each. Then, Cresyl Violet dye (Cresyl Violet Acetate, pure, high purity biological stain from Acros, cat# AC229630050) at a concentration of 0.2% was applied for 30 to 60 seconds, after which dehydration of the tissue was achieved by rinsing the slides with increasing concentrations of ethanol (50%, 75%, 95%, 100%, 100%) for 15 seconds each.

Laser Capture Microdissection

After the staining procedure, LCM was accomplished within 30 minutes using a Leica laser microscope (Leica LMD6500). Dissected stromal pieces were immediately collected in an RNAse/DNAse-free capture vial, containing 25µl of stabilizing RNA extraction buffer. During dissection, care was taken to avoid blood vessels, zones containing infiltrating immune cells, or fatty tissue. Dissection was restricted to fields contained within the perimeter of the invasive tumor, or at the perimeter of the tumor, but in direct relationship with invasive epithelial nests. Pictures were taken before and after the dissection procedure (see Figure 2 as an example). After finishing dissection for 1 tumor slide, 25 microliters of RNA extraction buffer were added into the capture vial, and lysis was performed for 30 minutes at 42°C. The obtained lysate was stored at -80°C until further RNA extraction.

For each patient, several tumor slides were laser dissected using this procedure (7 to 10 slides per patient according to size and amount of stromal fields within the tumoral tissue).

RNA extraction and amplification

RNA isolation was performed using the Arcturus Picopure RNA extraction kit (PicoPure™ RNA Isolation Kit, Arcturus, Cat # KIT0202/KIT0204) according to the manufacturer's protocol. Briefly, lysates from the same patient/tumor were combined, and after addition of 50 µl of ethanol 70%, the pooled samples were passed onto pre-conditioned RNA extraction columns. After centrifugation and washing, DNAse was applied onto the column to eliminate residual DNA (RNase-Free DNase Set from QIAGEN, cat# 79254). After washing, the purified RNA was eluted from the column using 11 µl of elution buffer. Samples were subsequently tested for RNA quality (RQI) on the Experion™ (Bio-Rad) using high sensitivity RNA chips, and concentrations were measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific). The quality of the RNA varied between samples, which is a known limitation of the laser capture microdissection procedure [38](see Supplementary Table 1). Prior to microarray analysis, RNA was pre-amplified using the Ovation PicoSL WTA System V2 (NuGEN, cat# 3312-24). The Ribo-SPIA technology implemented in this procedure is ideal for amplification of partially degraded and compromised RNA samples, contributes minimal coverage bias, and is highly reproducible[39]. The procedure is widely used in LCM projects and does not introduce significant bias into relative gene expression values[40,41]. A clean-up step using the Qiagen MinElute Reaction Cleanup Kit was also incorporated in the amplification procedure (Qiagen, cat#28204). After NuGEN pre-amplification of the RNA samples, RT-qPCR assessment of common housekeeping genes showed that the amplification procedure had resulted in highly concentrated cDNA fragments with sufficient size to be recognized by the primers (data not shown).

Gene expression analysis

Gene expression was analysed using the Affymetrix HG-U133Plus2 microarray chips at the J-C Heuson Breast Cancer Translational Research Laboratory (Jules Bordet Institute, Brussels, Belgium) following the manufacturer's instructions. Standard quality assessments were conducted on the resulting files, and all samples passed quality assurance for further analysis. Expression values were computed using the fRMA normalization method (fRMA Bioconductor package)[42] [PMID:20097884]. When multiple probe sets mapped to the same official gene symbol, we computed their average value.

The expression data are available on the GEO repository.

Statistical analysis

Differential expression analysis

To identify the genes that were differentially expressed in the two age categories (< 45 years vs ≥ 80 years), we computed for each probe set the mean expression value in both age groups and calculated the fold change of these means, i.e., the ratio of the average expression of this particular gene in young compared to old patients. Genes with fold change larger than 1.5 or smaller than -1.5 were considered as differentially expressed.

In silico validation

In order to validate the obtained differential gene expression data, gene expression data sets from other research projects that investigated laser dissected stromal samples obtained from breast cancer patients were retrieved. The datasets were available in NCBI Gene Expression Omnibus, under the following IDs: GEO:GSE5847; GEO:GSE4823; GEO:GSE14548.

Gene Set enrichment analysis (GSEA)

GSEA were conducted using our local reimplementation of the GSEA algorithm developed at the BROAD institute[43]. Briefly, genes were ranked according to their fold change in young versus old patients and an enrichment score (ES) ranging from -1 to 1 was computed. This score reflects to what extent the genes constituting a given reference class are enriched among the top up- or down-regulated genes of the differential expression analysis. Low (negative) ES values correspond to an enrichment of the reference class among genes that are up-regulated in old patients while high (positive) ES values correspond to an enrichment of the reference class among genes that are up-regulated in young patients. The FDR adjusted p-values associated with each ES value reflects the probability that an ES at least as high or as low could be obtained merely by chance. Adjusted p values <0.05 were considered as significant.

Results

Patient Demographics

For the purpose of the study, 17 female patients (9 young patients < 45 years at diagnosis, and 8 old patients ≥ 80 years at diagnosis) with available fresh-frozen breast cancer resection specimens and with sufficient stroma to allow laser-microdissection were selected. Extreme age categories were chosen in order to maximize the probability of detecting significant age-related differences. All patients underwent surgery for early breast cancer at the Multidisciplinary Breast Center (University Hospitals Leuven, Belgium) between 2000 and 2011. All patients had invasive ductal carcinomas of > 1.5 cm, and were negative for estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2. Additional patient and tumor characteristics are summarized in Table 1.

Differential Gene Expression Analysis

A differential gene expression analysis using a 1.5 fold up- or downregulation as cut-off, revealed 120 genes that were upregulated in older stromal samples and 107 genes that were downregulated in older stromal samples compared to younger (Table 2). Heatmaps constructed using the 25 top up- and downregulated genes, are shown in figure 3.

Data validation

As our sample size was small, we used publically available data sets in order to check the reproducibility of our findings. We found a significant overlap for 10 genes, of which 5 showed higher expression in older patients ($p<0.01$), and 5 genes showed a lower expression in older patients ($p<0.01$).

The Venn Diagrams depicting the overlapping genes are shown in figure 4; gene details are listed in table 3.

Gene Set enrichment analysis (GSEA)

As a second analytical approach, we looked at the expression of predefined gene sets documented in the literature to be related to specific processes. A GSEA was carried out for these candidate genes. The resulting enrichment score (ES), which ranges from -1 to 1, reflects the enrichment in genes of a given reference class among the top up- or down-regulated genes from the individual gene ranking. Plots are shown in figures 5 and 6. Negative ES values correspond to an enrichment of the reference class among genes that are upregulated in *old* patients while positive ES values correspond to an enrichment of the reference class among genes that are upregulated in *young*

patients. The genes that have been included in each GSEA, with respective literature references, are listed in Table 4.

Senescence genes

In the individual gene expression analysis, no significant difference was found for CDKN1A, CDKN2A, TP53, GLB1 nor the retinoblastoma (RB) gene. Nevertheless, the enrichment analysis for this gene set resulted in an enrichment score of -0.53, suggesting enrichment of senescence genes in the stroma of older patients, although statistical significance was not reached (p=0.09) (Fig 5B). The lack of significance might be due to the very small size of the reference classes.

DNA Damage response

None of the three most important components of the DNA damage response, ATM, NBN (=NBS1) and CHK2, were differentially expressed between young and old. Also, the obtained ES (0.57) for this gene set did not suggest upregulation in the older patients, and was not statistically significant (p=0.10) (Fig 5A).

Senescence Associated Secretory Profile

In the category of the genes involved in the Senescence Associated Secretory Profile, CXCL2 (fold change -1.59) and TNFRSF11B (fold change -2.11) were upregulated in the older patient samples, while CCL8 (fold change 1.61) showed downregulation in the older patient samples. The compiling GSEA analysis, though, confirmed a significant enrichment in SASP-related genes within the stroma of our older patient samples: ES -0.21; p=0.04 (Fig 6A).

The reverse Warburg effect – Autophagy genes

None of the genes described to be involved in the autophagy-senescence transition, showed a relevant difference in expression between young and old stroma at the individual gene level. However, when compiling them together in the GSEA analysis, we found a highly significant enrichment of autophagy genes in the older patient samples: ES -0.42; p<0.01 (Fig 6B).

Discussion

The reason for the age-related increase in cancers has been debated for decades. Besides cumulative DNA damage throughout life, the accumulation of senescent cells is also assumed to create a tumor-promoting microenvironment through the production of several cytokines, chemokines, and matrix remodeling enzymes (altogether called the SASP or Senescence Associated Secretory Profile). The SASP is held responsible for dedifferentiation, proliferation and increased migration/invasion of nearby (pre-)neoplastic cells[23,24]. Moreover, the autophagy to senescence transition (AST), a key pathophysiological process that seems to take place in senescent stromal cells, is thought to enhance tumor growth by producing high-energy mitochondrial fuels that boost epithelial cancer cell proliferation[33-35,44].

So far, studies investigating the impact of stromal senescence on tumor development were usually based on *in vitro* fibroblast cultures, where senescence had been artificially induced. The altered phenotype and functional activity of such senescent fibroblasts have been shown to influence the behaviour and growth speed of (pre)malignant cells, in cell cultures as well as in xenografts. However, an overload of senescent or presenescent fibroblasts was present in these experiments, whereas solid data on the *in vivo* abundance of senescent fibroblasts in the older breast are lacking. I*n vivo* senescence has mostly been studied in fibroblasts localized in the skin[14]. Furthermore, there is only limited clinical support for a more aggressive behaviour of breast cancers in older patients, even when correcting for histopathological characteristics[37]. This is in contradiction with the hypothesis that senescent stromal fibroblasts would promote tumor growth and invasion. We therefore wanted to investigate the molecular footprint of the older breast cancer microenvironment, in order to find *in vivo* confirmation of key concepts such as aging/senescence, DNA Damage Response, SASP, and AST. For this purpose, we selected two groups of patients belonging to extreme age categories, isolated cancer-associated stromal fields via LCM and investigated the gene expression profile.

Differential gene expression analysis using a cut-off at 1.5 fold change in expression revealed 120 upregulated and 107 downregulated genes in the stromal parts of older patients, compared to the younger ones. Validation of these findings on publically available stromal data, revealed a set of ten differentially expressed genes between young and old stromal samples. The *young* stromal samples mainly showed upregulation in genes that preclude migration and invasion by stabilizing the cells in the extracellular matrix, and stimulate differentiation (see Table 3 and Figure 4). *RARRES 3* is a gene that regulates adhesion and differentiation of cells. In the context of tumoral cells, it has been

described as tumor suppressive[45-47]. *RARRES3* has been described to have a suppressive effect on the development of metastases in colorectal cancer[48] as well as in breast cancer[45,49]. It is positively correlated with tumor differentiation in colorectal cancer[50], and downregulated at disease progression in B cell lymphocytic leukemias[51]. Likewise, *SCUBE2* is a gene that inhibits breast cancer cell migration and invasion[52,53]. The clinicopathological correlations of *SCUBE2* have been investigated in colorectal cancer, where expression of *SCUBE2* was shown to correlate with a better outcome of the disease: Patients with *SCUBE2*-positive tumors had a lower recurrence rate and better survival than patients with *SCUBE2*-negative tumors[54]. Also in breast cancer, Patients with positive *SCUBE2* protein-expressing tumors have been shown to have better prognosis than those with negative *SCUBE2* protein-expressing tumors in terms of disease-free survival[55]. A prognostic value of *SCUBE2* in endometrial cancer has also been proposed: *SCUBE2* expression was shown to be higher in grade 3 tumors versus grade 1 endometrial cancers. A positive correlation of *SCUBE2* expression with expression of hormone receptors (ER and PR) and PTEN was shown suggesting that *SCUBE2* is a positive prognostic factor also in endometrial cancer[56]. Upregulation of *SFRP4*, a component of the Wnt signalling pathway, improves adherence of cells to surrounding collagen and fibronectin, and thus results in less migration through the extracellular matrix[57]. *SFRP4* has been shown to enhance sensitivity of cancer cells to chemotherapy[58-60]. It is the gatekeeper/inhibitor of Wnt signaling, and has been shown to decrease stem cell properties in cancer cells, to reverse epithelial to mesenchymal transition[61,62] thereby leading to a less aggressive cell phenotype[10] and a better outcome. *COMP* encodes a noncollagenous extracellular matrix protein. It is involved in the chondrogenic differentiation of mesenchymal stem cells.[63,64] *COMP* has been studied in breast cancer cells, where high expression was correlated with shorter disease free survival and poor survival. In mice experiments, tumors expressing *COMP* were significantly larger and were more prone to metastasize as compared with control, mock-transfected, tumors. The authors found that *COMP*-expressing tumor cells appeared to undergo a metabolic switch, that is, a Warburg effect[65]. The molecular role of *NAT1* is not very clear. Its major role seems metabolisation of xenobiotics. *NAT1* expression has been studied in breast cancer where higher expression has been associated with better disease free and overall survival[66] in women, but also with better overall survival in male breast cancer[67].

Taken together, it seems like the younger stromal compartment shows more signs of a stable extracellular matrix, where cells have a lower migrating capacity. Only for *COMP*, the compatibility with this hypothesis is less clear. An explanation could be that the outcome related to overexpression of a gene can differ according to the type of cell in which it is overexpressed: tumor cells versus stromal cells.

Significant upregulation in the *older* stromal microenvironment was shown for genes that are involved in proliferation, dedifferentiation and angiogenesis. *ANXA3* has been associated with poor prognosis and increased proliferation rates in malignant disease. It is involved in the regulation of apoptosis, by affecting the Bcl-2/Bax-balance. Positive correlations with tumor size, axillary lymph node metastasis, and a worse overall survival in breast cancer have been reported[68]. When *ANXA3* is inhibited, cell proliferation is decreased[68-70]. Elevated *ANXA3* expression has also shown to be associated with tumor size, number of lesions, tumor stage, and poor prognosis in hepatocellular carcinoma[71]. A correlation with the development of colorectal adenocarcinoma has been suggested[72]. In renal cell carcinoma, the relationship seems opposite: downregulation of *ANXA3* was shown in renal cell cancers (versus non tumoral cells)[73] although further distinction is made between expression of the long and short isoforms of the protein. *PROM1*, also called *CD133*, is the second upregulated gene in the older patient samples. It is a transmembrane glycoprotein expressed by adult stem cells and thought to maintain stem cell properties by suppressing differentiation. It has mainly been described in tumors (hepatocellular carcinoma, melanoma), where it correlates with bad outcome measures such as advanced disease, more aggressive disease and metastatis [74-77]. Also in small cell lung cancer, higher expression of *PROM1* is linked to a worse prognosis (survival)[78]. In paediatric medulloblastoma, a significantly higher expression of *PROM1* was found both in patients with poorer prognosis as in those with metastasis. Kaplan-Meier analysis showed that both overall survival (OS) and progression-free survival (PFS) were shorter in patients with higher *PROM1* mRNA levels than in patients with lower expression[79]. *FGF13* plays a role in a variety of biological processes. It is a mitogenic, angiogenic[80] and survival factor involved in cell migration and cell differentiation[81]. A high cytoplasmic staining of *FGF13* was shown to be associated with a higher risk of biochemical recurrence (BCR) in prostate cancer, after radical prostatectomy[82]. High *FGF13* expression was also suggested to mediate resistance of cancer cells to platinum based chemotherapy in cervical cancer cells[83]. *TUBB2B* was also upregulated in the older stromal samples, but its implication in the present context is less clear. The protein encoded by this gene is a major component of microtubules and has mainly been described in association with brain malformations[84]. In accordance with the mechanism of action of taxane-chemotherapy, the expression of *TUBB2B* has been described as a predictive marker of docetaxel activity[85]. A higher expression of *TUBB2B* in non-small cell lung cancer was associated with increased disease aggressiveness [86]. In melanoma, microarray analysis showed that the β-tubulin gene group was significantly upregulated in a subpopulation with higher metastatic potential. *TUBB2B* which is a member of this group, exhibited significantly enhanced expression[87].

Thus, these four genes, with upregulated expression in the older stromal samples, all seem to restrain differentiation and promote cell proliferation, invasiveness and metastasis, as opposed to the function of 4 of the 5 genes upregulated in young stromal samples. The fifth upregulated gene in the older stroma samples, *WIF1*, is a negative inhibitor of the Wnt(Wingless-type)/β-catenin signalling pathway. It is thought to inhibit proliferation, induce differentiation and cellular senescence by upregulation of tumor suppressor genes such as p53 or p21[88,89]. It is expressed by stem cells of the human interfollicular epidermis, and acts to suppress keratinocyte proliferation[89]. *WIF1* protein expression in colorectal cancer has shown to be increased in cancer tissue versus normal tissue, and high expression of *WIF1* showed to be associated with big tumor diameters and deep invasion[90]. On the contrary, *WIF1* hypermethylation (i.e. silencing of the gene) was shown to be associated with an unfavorable prognosis in EGFR mutated lung adenocarcinoma patients[91], and associated with ovarian cancers[92] and cervical cancer[93]. While the proliferation-inhibiting and differentiation-inducing effects of this gene seem to be in contradiction with the proliferation- and metastasis-promoting activity of the other four upregulated genes in the older stromal samples, its senescence-inducing function may be an obvious explanation for the age-related stromal expression of *WIF1* in our study.
Taken together, we found evidence of a more tumor-favorable micro-environment in the stromal samples from older patients.

As an additional analysis, we applied a candidate-gene approach by assembling sets of genes based on the literature. We specifically looked at the individual gene expression results for these genes, but also compiled them using a gene set enrichment strategy that reflects the representation of these genes among the top up- or down-regulated genes in old and young stroma samples.

The molecular process of senescence is characterized by upregulation of several senescence genes. The most documented ones are *TP53*, *CDKN2A (p16)*, *CDKN1A (p21)* and *pRB*[94-96].
These major senescence-inducing genes did not show significantly different expression values between young and old stroma in the individual gene analysis. Nevertheless, we observed in older patients an upregulation of PAI-1 (=SERPINE1, fold change -1.87), a matrix remodeling enzyme, which has also been described as a crucial regulator of aging and senescence by acting downstream of p53 and upstream of insulin-like growth factor binding protein-3[97]. Furthermore, WIF1, described above as inhibitor of the Wnt/β-catenin signalling pathway and an inducer of senescence, was also increased in samples from old compared to young patients (fold change -2.10) (Table 2). These findings could be indicative of more widespread cellular senescence in our older stromal samples, compared to the young ones. Yet, as we have selected extreme age categories, we actually

had expected more pronounced differences in typical markers of cellular senescence like p16 or p53, which, however, remained unchanged. Gene enrichment analysis, based on 5 key senescence genes (see table 4) including the ones mentioned above, showed a tendency towards a more prominent senescence trait in older stroma, but no significant enrichment score was reached for this process in the older patient samples. Therefore we cannot decisively conclude that samples from the older patient group show increased senescence.

The DNA damage response is a biological process that, upon severe DNA damage, triggers the switch towards a permanent growth arrest[98]. It was found that the molecular senescence program can only be induced when this DNA damage response has been activated for a sufficiently long time period[21].
We could not demonstrate any clear difference in the individual expression of 3 key players involved in the DNA damage response (ATM, NBN or CHEK2)[98], nor did we find significant enrichment for this set of genes in the older patient samples.

Coppé et al compared cell culture supernatants from senescent versus non-senescent cell cultures[26], and observed a marked overproduction and secretion of senescence-associated factors in the senescent cell cultures. The authors confirmed that these changes were due to upregulation of gene expression. In our stromal gene expression study, only a few of the SASP components described by Coppé et al. showed significant age-related differential expression: CXCL2, a chemokine with immunoregulatory and inflammatory capacities, and osteoprotegerin (TNFRSF11B), a member of the TNF receptor superfamily showed overexpression in the old patient samples, while CCL8 showed downregulation in the older versus the younger stromal samples.
However, when compiling all the components of the SASP together in the gene enrichment analysis, we indeed confirmed a significant enrichment in SASP genes among genes upregulated in the older patient samples. SASP components are believed to act in a paracrine way, thereby influencing the surrounding cells. Premalignant cells, neighboured by senescent fibroblasts, lose differentiated properties, become invasive and undergo full malignant transformation[20,23-25]. Malignant breast epithelial cells, co-injected with senescent fibroblasts into xenografts, give rise to tumors much more rapidly compared to malignant cells alone[24,29]. In non-malignant breast epithelial cells, senescent fibroblasts have been shown to disrupt the epithelial alveolar morphogenesis, the functional differentiation and the branching morphogenesis[23]. Also, the inflammatory microenvironment of the aging prostate has been suggested to stimulate the proliferation of both epithelial cells and fibroblasts[30]. Furthermore, older stromal cells, when cultured in vitro, were shown to promote tumor formation from epithelial cell lines when co-

injected in mice[31]. Here, we confirm for the first time the presence of the 'SASP' phenomenon in spontaneous, human breast cancers.

Autophagy is assumed to precede or parallel the process of senescence[33], as described by the term Autophagy to Senescence transition (AST). Typical markers for AST are loss of Caveolin-1 (CAV1), upregulation of BNIP3, BNIP3L, Beclin-1, Cathepsine B, and ATG16L1.
Our individual gene expression results did not show relevant upregulation of single autophagy-related genes in the older stromal samples, but compilation of these genes into an enrichement analysis showed highly significant enrichment for autophagy genes in the older stromal samples. Thus, in addition to the presence of SASP, we also confirmed the presence of AST in the older stromal cancer milieu. As described above, AST promotes the production of high-energy metabolites, fueling the growth of nearby cancer cells.

The fact that we confirm the presence of SASP and AST in older stromal samples provides *in vivo* support for the *in vitro* and xenograft evidence on these phenomena. This, however, does not solve the paradox between the stimulatory effect that these processes are supposed to have on proximate malignant cells, and the clinical finding that breast cancer in older patients rather behaves in a more indolent instead of a more aggressive way[99]. Also, it remains puzzling that we find evidence for SASP and AST in older stromal samples, while we do not find convincing evidence for increased senescence in these samples. The small sample size, together with the low number of genes defining the 'senescence' program could partly explain the lack of significance for major senescence-related genes such as TP53, CDKN2A and pRB, both at the individual level and in the gene enrichment analysis. Further analysis like staining for ϒ-H2AX foci could bring additional information on this question.

Further *in vivo* work on this topic is certainly required. The technical challenge to dissect stroma from invasive tumor makes it difficult to study larger patient groups. However, *in vivo* confirmation of *in vitro* discoveries is indispensable to ensure constructive research efforts.

Conclusion

For the first time, we report on key pathophysiological concepts of cancer and aging, such as the Senescence Associated Secretory Profile, and the Autophagy to Senescence transition, in vivo in human cancer patients.
Our data, obtained by laser microdissection of breast cancer stroma from tumors diagnosed in young and old patients, show a modest, but significant difference in gene expression in young

versus old stromal samples. The difference concerns mainly genes responsible for proliferation, differentiation and migration into the extracellular matrix.
Moreover, gene enrichment analysis confirms the presence of a Senescence Associated Secretory Profile and Autophagy to Senescence transition in the older stromal compartments, which to date, had never been investigated in spontaneous human cancerous lesions. These remarkable findings justify further research to fully elucidate the role of the aging stroma in (breast) tumor development and progression.

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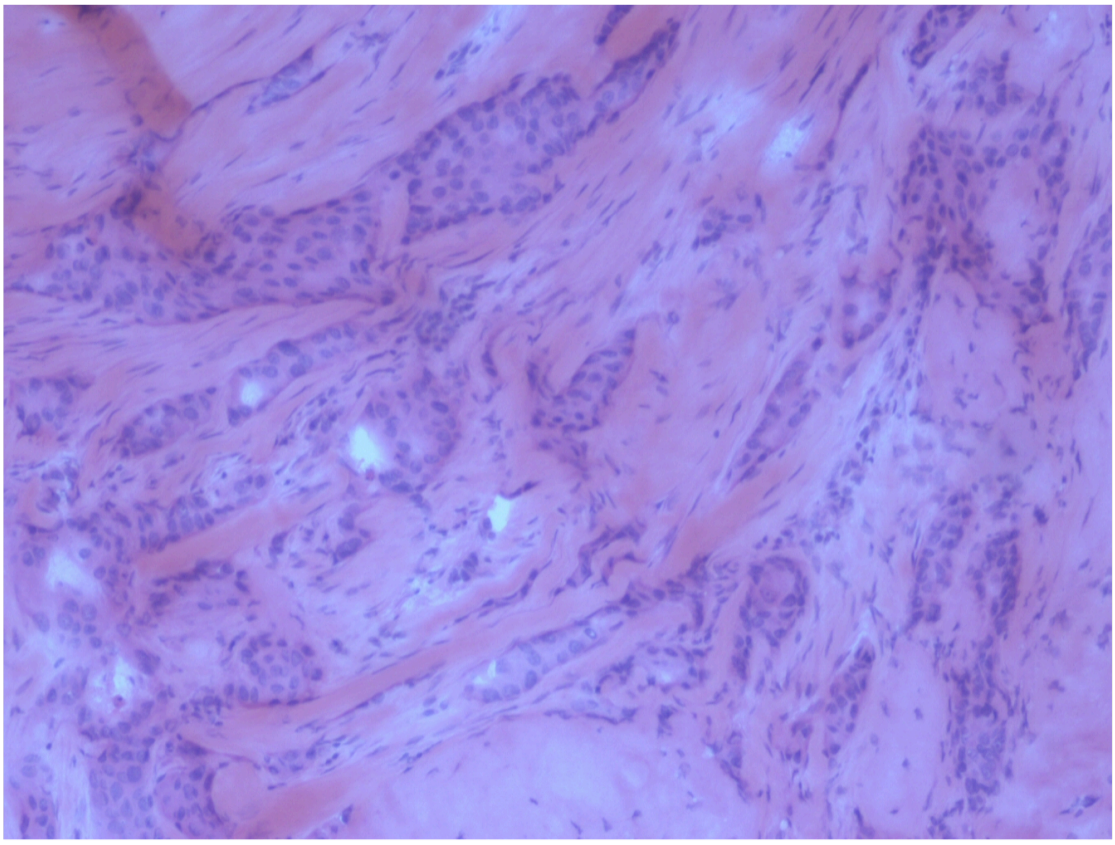

Figure 1 : H&E image of selected tumor block for Laser Capture Microdissection

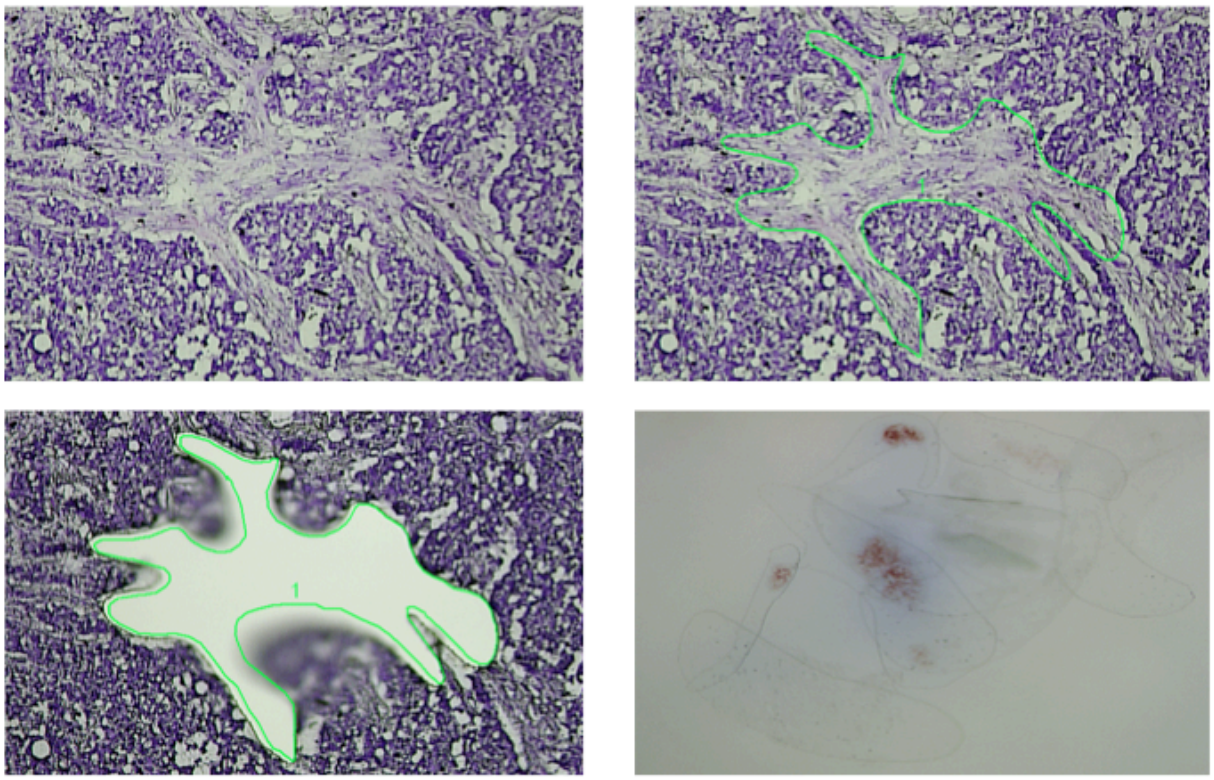

Figure 2 : laser Capture Microdissection of cancer associated stroma. Panal A-B-C show the microdissection procedure, panel D shows the yield of stromal pieces after repeated microdissection within the same tumor slide

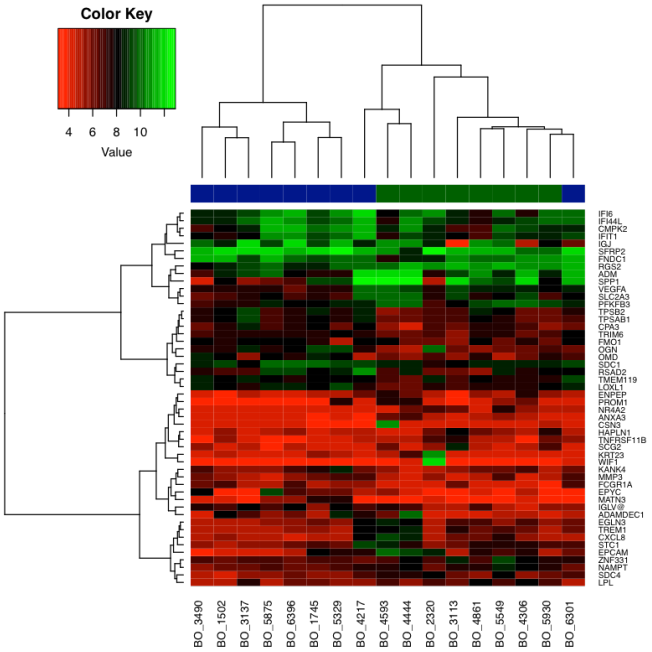

Figure 3 : heatmaps constructed using the 25 top up-regulated and the 25 top down-regulated genes. Blue patients are young patients, green patients are old patients

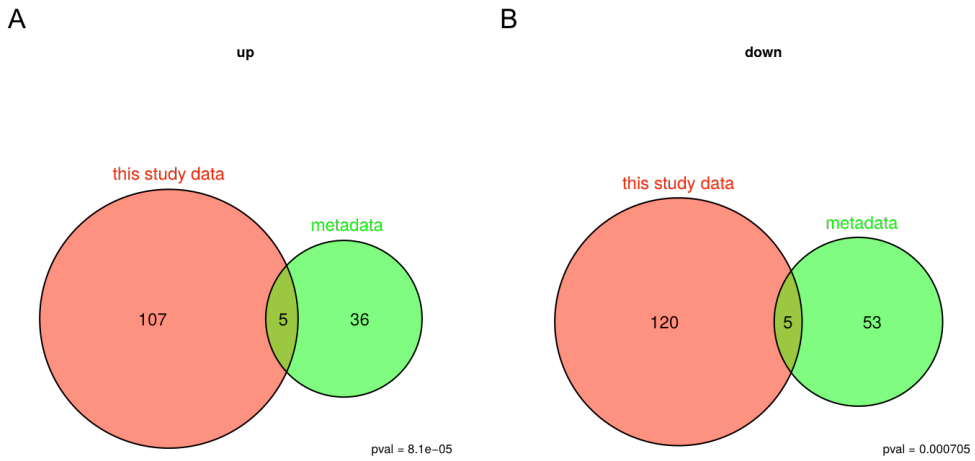

Figure 4 : Venn Diagrams showing the intersection of our own expression data (called ‘Study data’ and the publically available data (called ‘Metadata’). Panel A shows the upregulated genes in young stromal samples, Panel B shows the downregulated genes in young stromal samples

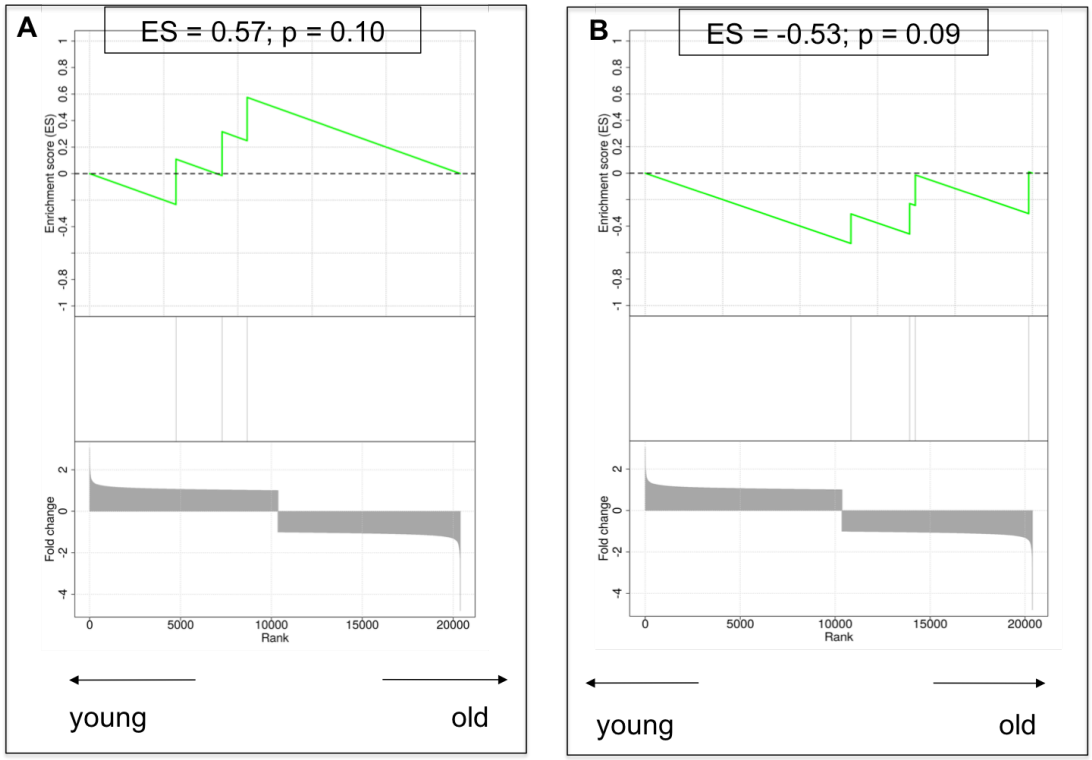

Figure 5: GSEA (gene enrichment analysis) plots. Panel A shows the process of DNA Damage Response, Panel B shows Senescence Genes. ES: Enrichment Score, low values correspond to enrichment of the genes in older, high values to enrichment of the genes in young patients

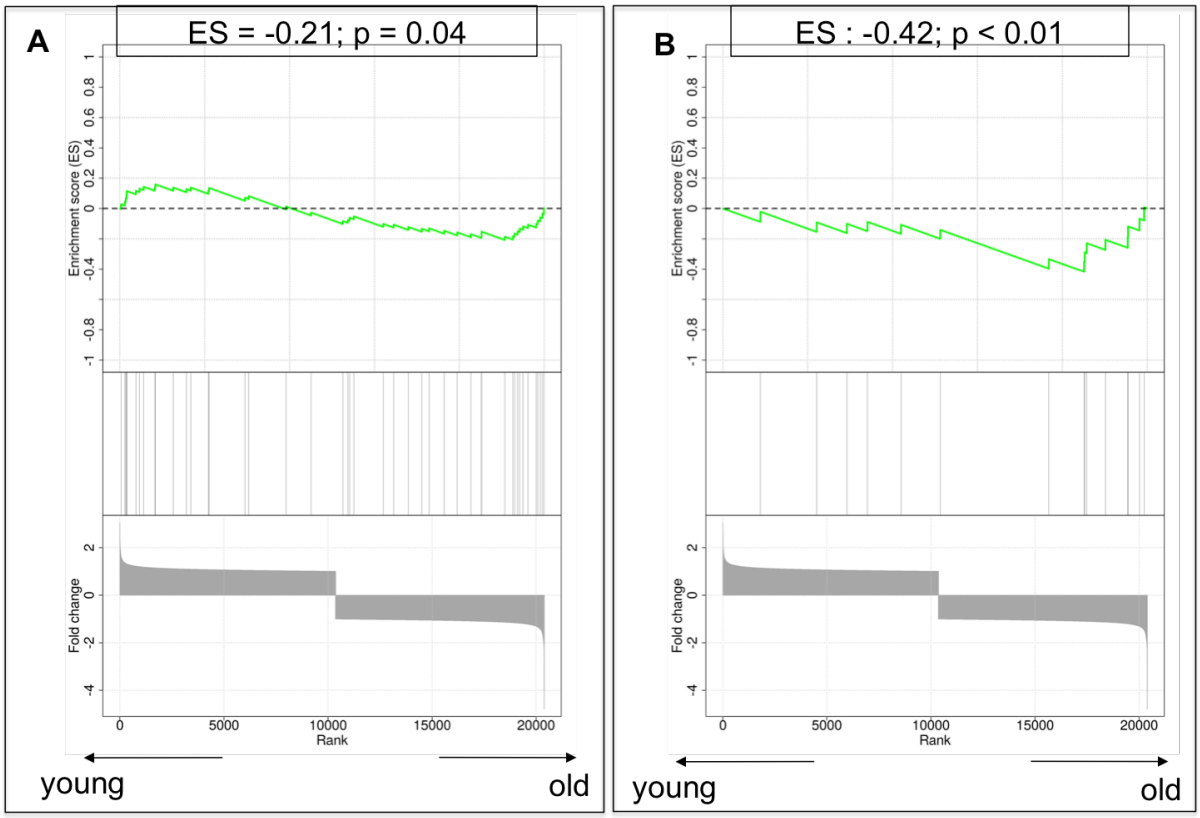

Figure 6: GSEA (gene enrichment analysis) plots. Panel A shows the process of Senescence Associated Secretory Profile, Panel B shows the Autophagy to Senescence transition – or Reverse Warburg Effect. . ES: Enrichment Score, low values correspond to enrichment of the genes in older, high values to enrichment of the genes in young patients

Patient ID	age at diagnosis	ER	PR	HER2	Tumor Type	Tumor grade	Maximum tumor size (cm)	pT-stage	pN-stage
6	27	neg	neg	neg	ductal	3	2.3	2	0
5	30	neg	neg	neg	ductal	3	2.5	2	0
7	32	neg	neg	neg	ductal	3	2.2	2	0
1	33	neg	neg	neg	ductal	3	2.8	2	0
3	39	neg	neg	neg	ductal	3	3.0	2	0
2	43	neg	neg	neg	ductal	3	3.0	2	2a
4	44	neg	neg	neg	ductal	3	2.8	2	0
8	44	neg	neg	neg	ductal	3	3.5	2	0
9	44	neg	neg	neg	ductal	3	3.0	2	0
12	80	neg	neg	neg	ductal	3	4.0	2	0
16	82	neg	neg	neg	ductal	3	3.5	2	0
17	82	neg	neg	neg	ductal	3	1.5	1c	0
13	82	neg	neg	neg	ductal	2	3.0	2	3a
15	83	neg	neg	neg	ductal	3	3.8	2	1a
10	83	neg	neg	neg	ductal	3	3.2	2	0
11	86	neg	neg	neg	ductal	3	3.0	2	0
14	87	neg	neg	neg	ductal	3	2.0	1c	0

Table 1 : Patient and tumor characteristics (pT and pN stands for pathological T and N stage following the TNM staging)

Gene	Full Name	Fold Change
SPP1	secreted phosphoprotein 1	-4,79
EPCAM	epithelial cell adhesion molecule	-4,02
IL8	Interleukin 8	-2,74
NR4A2	nuclear receptor subfamily 4, group A, member 2	-2,45
RGS2	regulator of G-protein signaling 2, 24kDa	-2,41
TREM1	triggering receptor expressed on myeloid cells 1	-2,36
PROM1	prominin 1	-2,27
SCG2	secretogranin II	-2,22
LPL	lipoprotein lipase	-2,20
SDC4	syndecan 4	-2,19
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	-2,13
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	-2,11
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	-2,11
WIF1	WNT inhibitory factor 1	-2,10
NAMPT	nicotinamide phosphoribosyltransferase	-2,08
ENPEP	glutamyl aminopeptidase (aminopeptidase A)	-2,07
ZNF331	zinc finger protein 331	-2,07
ANXA3	annexin A3	-2,06
HAPLN1	hyaluronan and proteoglycan link protein 1	-2,05
CSN3	casein kappa	-2,05
KRT23	keratin 23 (histone deacetylase inducible)	-2,05
VEGFA	vascular endothelial growth factor A	-2,03
STC1	stanniocalcin 1	-2,01
EGLN3	egl nine homolog 3 (C. elegans)	-1,97
ADM	adrenomedullin	-1,96
G0S2	G0/G1switch 2	-1,95
BAMBI	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	-1,93
TDO2	tryptophan 2,3-dioxygenase	-1,93
CD24	CD24 molecule	-1,92
DNER	delta/notch-like EGF repeat containing	-1,92
IBSP	integrin-binding sialoprotein	-1,91
HSPA2	heat shock 70kDa protein 2	-1,90
ERRFI1	ERBB receptor feedback inhibitor 1	-1,89
MUCL1	mucin-like 1	-1,89
APOLD1	apolipoprotein L domain containing 1	-1,89
SHISA2	shisa homolog 2 (Xenopus laevis)	-1,88
GPX3	glutathione peroxidase 3 (plasma)	-1,87
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-1,87
COL2A1	collagen, type II, alpha 1	-1,86
CP	ceruloplasmin (ferroxidase)	-1,85
COL9A3	collagen, type IX, alpha 3	-1,85
ENO2	enolase 2 (gamma, neuronal)	-1,84
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	-1,84

TSPAN13	tetraspanin 13	-1,82
CYP4X1	cytochrome P450, family 4, subfamily X, polypeptide 1	-1,82
TFAP2C	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)	-1,81
EGR3	early growth response 3	-1,81
SOX11	SRY (sex determining region Y)-box 11	-1,79
CLEC5A	C-type lectin domain family 5, member A	-1,78
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	-1,78
SLPI	secretory leukocyte peptidase inhibitor	-1,78
PI15	peptidase inhibitor 15	-1,78
RBP7	retinol binding protein 7, cellular	-1,77
SERPINA3	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	-1,77
CCDC102B	coiled-coil domain containing 102B	-1,75
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	-1,74
CFI	complement factor I	-1,74
FCGBP	Fc fragment of IgG binding protein	-1,73
GPNMB	glycoprotein (transmembrane) nmb	-1,73
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	-1,72
MAL2	mal, T-cell differentiation protein 2	-1,72
UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1	-1,71
IER3	immediate early response 3	-1,70
COL4A1	collagen, type IV, alpha 1	-1,69
EFNB2	ephrin-B2	-1,69
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)	-1,69
BTBD3	BTB (POZ) domain containing 3	-1,68
FGF13	fibroblast growth factor 13	-1,68
GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	-1,67
INHBB	inhibin, beta B	-1,66
MANSC1	MANSC domain containing 1	-1,65
DSP	desmoplakin	-1,64
CLDN8	claudin 8	-1,64
TUBB2B	tubulin, beta 2B	-1,64
PODXL	podocalyxin-like	-1,63
EHF	ets homologous factor	-1,63
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	-1,63
ANGPT2	angiopoietin 2	-1,62
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	-1,62
GPR4	G protein-coupled receptor 4	-1,61
DBH	dopamine beta-hydroxylase (dopamine beta-monooxygenase)	-1,61
GPR183	G protein-coupled receptor 183	-1,61
TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	-1,60
SNORD89	small nucleolar RNA, C/D box 89	-1,60
CXCL2	chemokine (C-X-C motif) ligand 2	-1,60

CXADR	coxsackie virus and adenovirus receptor	-1,60
TPRKB	TP53RK binding protein	-1,60
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	-1,60
RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	-1,60
ADGRF5	adhesion G protein-coupled receptor F	-1,60
CA2	carbonic anhydrase II	-1,59
LIPA	lipase A, lysosomal acid, cholesterol esterase	-1,59
PGM2	phosphoglucomutase 2	-1,59
KRT19	keratin 19	-1,58
MGAT5	mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase	-1,58
NCF2	neutrophil cytosolic factor 2	-1,57
RHOU	ras homolog gene family, member U	-1,57
ALCAM	activated leukocyte cell adhesion molecule	-1,57
LRRN1	leucine rich repeat neuronal 1	-1,57
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	-1,55
SLC19A2	solute carrier family 19 (thiamine transporter), member 2	-1,55
PRPS2	phosphoribosyl pyrophosphate synthetase 2	-1,55
MEGF10	multiple EGF-like-domains 10	-1,55
CYYR1	cysteine/tyrosine-rich 1	-1,54
PLVAP	plasmalemma vesicle associated protein	-1,54
TM4SF1	transmembrane 4 L six family member 1	-1,54
PDGFA	platelet-derived growth factor alpha polypeptide	-1,54
YBX2	Y box binding protein 2	-1,54
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	-1,54
PCDHB2	protocadherin beta 2	-1,54
DNMT1	DNA (cytosine-5-)-methyltransferase 1	-1,54
S100A8	S100 calcium binding protein A8	-1,53
MAP2	microtubule-associated protein 2	-1,53
ARRDC4	arrestin domain containing 4	-1,52
FAM83D	family with sequence similarity 83, member D	-1,52
LSR	lipolysis stimulated lipoprotein receptor	-1,52
STK26	serine/threonine protein kinase 26	-1,51
MIR181A2HG	MIR181A2 host gene (non-protein coding)	-1,51
VWA8	von Willebrand factor A domain containing 8	-1,51
MEST	mesoderm specific transcript homolog (mouse)	-1,51
ZNF835	zinc finger protein 835	1,51
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	1,51
EPSTI1	epithelial stromal interaction 1 (breast)	1,51
LOC221946	hypothetical LOC221946	1,51
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	1,52
SELL	selectin L	1,52
COX6C	cytochrome c oxidase subunit VIc	1,52
TRIM41	tripartite motif-containing 41	1,52
IFI27	interferon, alpha-inducible protein 27	1,52
IGF1	insulin-like growth factor 1 (somatomedin C)	1,52
SCAMP1-AS1	SCAMP1 antisense RNA 1	1,52

CD207	CD207 molecule, langerin	1,52
IFI35	interferon-induced protein 35	1,52
GGH	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	1,52
NOX4	NADPH oxidase 4	1,53
CNTN3	contactin 3 (plasmacytoma associated)	1,53
CCL5	chemokine (C-C motif) ligand 5	1,54
GALNT1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	1,54
SPON1	spondin 1, extracellular matrix protein	1,54
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	1,54
DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	1,55
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	1,55
CXCL14	chemokine (C-X-C motif) ligand 14	1,55
WISP2	WNT1 inducible signaling pathway protein 2	1,55
STAT1	signal transducer and activator of transcription 1, 91kDa	1,55
COMP	cartilage oligomeric matrix protein	1,56
IGLJ3	immunoglobulin lambda joining 3	1,56
LRRC17	leucine rich repeat containing 17	1,56
IFI44	interferon-induced protein 44	1,56
ISG15	ISG15 ubiquitin-like modifier	1,56
FBLN2	fibulin 2	1,57
SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	1,57
MX2	myxovirus (influenza virus) resistance 2 (mouse)	1,57
SH3D19	SH3 domain containing 19	1,57
TRBC1	T cell receptor beta constant 1	1,58
SGCE	sarcoglycan, epsilon	1,58
IGHM	immunoglobulin heavy constant mu	1,58
DCBLD1	discoidin, CUB and LCCL domain containing 1	1,59
PPAPDC1A	phosphatidic acid phosphatase type 2 domain containing 1A	1,59
BST2	bone marrow stromal cell antigen 2	1,59
MFAP2	microfibrillar-associated protein 2	1,60
PDGFD	platelet derived growth factor D	1,60
IGKC	immunoglobulin kappa constant	1,60
CST1	cystatin SN	1,61
CCL8	chemokine (C-C motif) ligand 8	1,61
RASGRF2	Ras protein-specific guanine nucleotide-releasing factor 2	1,61
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	1,63
PDGFRL	platelet-derived growth factor receptor-like	1,63
ALDH1L2	aldehyde dehydrogenase 1 family, member L2	1,63
FAM198B	family with sequence similarity 198, member B	1,63
MIR100HG	mir-100-let-7a-2 cluster host gene	1,64
GAPT	GRB2-binding adaptor protein, transmembrane	1,65
SELM	selenoprotein M	1,65

DSCAM-AS1	DSCAM antisense RNA 1	1,66
STMN2	stathmin-like 2	1,69
FBLN5	fibulin 5	1,70
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	1,70
SFRP4	secreted frizzled-related protein 4	1,71
ACKR4	atypical chemokine receptor 4	1,71
CPNE2	copine II	1,71
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	1,72
ST6GAL2	ST6 beta-galactosamide alpha-2,6-sialyltranferase 2	1,72
NEXN	nexilin (F actin binding protein)	1,72
CD52	CD52 molecule	1,72
MFAP5	microfibrillar associated protein 5	1,73
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	1,75
GXYLT2	glucoside xylosyltransferase 2	1,75
HMCN1	hemicentin 1	1,76
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	1,78
IL21R	interleukin 21 receptor	1,78
C8orf4	chromosome 8 open reading frame 4	1,78
LINC01503	long intergenic non-protein coding RNA 1503	1,78
OLFML3	olfactomedin-like 3	1,79
CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	1,81
MVB12A	multivesicular body subunit 12A	1,82
SCUBE2	signal peptide, CUB domain, EGF-like 2	1,83
WNT2	wingless-type MMTV integration site family member 2	1,85
APOL3	apolipoprotein L, 3	1,87
ADRA2A	adrenergic, alpha-2A-, receptor	1,89
HIST1H3I	histone cluster 1, H3i	1,92
SLC46A3	solute carrier family 46, member 3	1,92
ARHGAP28	Rho GTPase activating protein 28	1,93
KANK4	KN motif and ankyrin repeat domains 4	1,93
SDC1	syndecan 1	1,95
CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	1,96
IFI44L	interferon-induced protein 44-like	1,97
FMO1	flavin containing monooxygenase 1	1,98
TMEM119	transmembrane protein 119	1,99
FNDC1	fibronectin type III domain containing 1	2,00
ADAMDEC1	ADAM-like, decysin 1	2,00
TPSAB1	tryptase alpha/beta 1	2,02
CPA3	carboxypeptidase A3 (mast cell)	2,02
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	2,05
IFI6	interferon, alpha-inducible protein 6	2,06
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	2,06
SFRP2	secreted frizzled-related protein 2	2,09
TRIM6	tripartite motif-containing 6	2,10
TPSB2	tryptase beta 2 (gene/pseudogene)	2,19

RSAD2	radical S-adenosyl methionine domain containing 2	2,28
LOXL1	lysyl oxidase-like 1	2,30
OMD	osteomodulin	2,35
IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	2,44
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	2,47
MATN3	matrilin 3	2,55
IGLV@	immunoglobulin lambda variable cluster	2,65
OGN	osteoglycin	2,99
EPYC	epiphycan	3,04

Table 2 : Genes with >1.5 or < -1.5 fold expression and respective fold changes. Negative values for fold change indicate upregulation in older patient samples, positive values indicate upregulation in younger patient samples.

Gene	Full Name	Fold change
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	1.75
SFRP4	secreted frizzled-related protein 4	1.71
SCUBE2	signal peptide, CUB domain, EGF-like 2	1.83
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	1.51
COMP	cartilage oligomeric matrix protein	1.56
ANXA3	annexin A3	-2.06
PROM1	prominin 1	-2.27
FGF13	fibroblast growth factor 13	-1.68
TUBB2B	tubulin, beta 2B	-1.64
WIF1	WNT inhibitory factor 1	-2.10

Table 3: Significant up- or downregulated genes after validation in the external validation dataset (see Fig 4). Negative values for fold change indicate upregulation in old patient samples, positive values indicate upregulation in young patient samples.

Gene Group	Involved Genes	References
Senescence Associated Secretory profile	IL1A, IL6, IL6R, IL6ST, IL8, CXCL1, CXCL2, CXCL3, CSF2, IL7, ICAM1, TNFRSF11B, HGF, IGFBP4, CCL8, PLAUR, IGFBP2, CCL26, IL13, CCL20, ICAM3, PGF, TNFRSF1A, TNFRSF1B, CCL13, CCL16, TNFRSF10C, CCL2, FAS, ANG, IGFBP6, IL1B, (CCL3), TIMP2, IL11, OSM, LEP, AXL, KITLG, FGF7, IL15, FGF2, IGFBP1, MIF	(17) (21 - 22) (26)
Autophagy to Senescence Transition	CAV1, CTSB, BNIP3, PRKAA1, PRKAA2, LAMP2, MAP1LC3B, ATG16L1, HIF1A, NFKB1, DRAM1, TP73, MAPK8, E2F1, STK11	(33 - 36) (44)
DNA Damage Response	ATM, NBN, CHEK2	(21) (98)
Cellular Senescence	CDKN1A, CDKN2A, TP53, RB1, GLB1	(17 – 19) (94 - 96)

Table 4: Groups of candidate genes related to a specific pathophysiological process, built to perform gene set enrichment analysis (GSEA), and their respective references.

Patient ID	RNA concentration before amplification (ng/microliter)	RQI	RNA concentration after amplification (ng/microliter)
1 $	4,6	3,9	340,8
2 $	7,7	6,9	441,7
3 $	3,4	na	498,8
4 $	9,2	4,6	361,8
5 $	7,2	5	328,9
6 $	3,8	4,8	344,5
7 $	5,8	6,7	439,2
8 $	4,9	2,2	410,8
9 $	3,9	na	388,3
10 δ	12,3	7,7	458,6
11 δ	9,7	6	482,0
12 δ	10,7	6,5	577,1
13 δ	6,6	6,6	338,9
14 δ	7,5	6,8	346,3
15 δ	8,8	3,9	266,2
16 δ	4,5	7,0	448,7
17 δ	8,0	2,7	465,0

$ patient belongs to the young patient group
δ patient belongs to the older patient group
na : not available

Supplementary Table 1 : RNA concentration and RQI value (RNA Quality Indicator) before, and RNA concentration after amplification

Research Paper

Biological ageing and frailty markers in breast cancer patients

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Abstract: Older cancer patients are a highly heterogeneous population in terms of global health and physiological reserves, and it is often difficult to determine the best treatment. Moreover, clinical tools currently used to assess global health require dedicated time and lack a standardized end score. Circulating markers of biological age and/or fitness could complement or partially substitute the existing screening tools. In this study we explored the relationship of potential ageing/frailty biomarkers with age and clinical frailty. On a population of 82 young and 162 older non-metastatic breast cancer patients, we measured mean leukocyte telomere length and plasma levels of interleukin-6 (IL-6), regulated upon activation, normal T cell expressed and secreted (RANTES), monocyte chemotactic protein 1 (MCP-1), insulin-like growth factor 1 (IGF-1). We also developed a new tool to summarize clinical frailty, designated Leuven Oncogeriatric Frailty Score (LOFS), by integrating GA results in a single, semi-continuous score. LOFS' median score was 8, on a scale from 0=frail to 10=fit. IL-6 levels were associated with chronological age in both groups and with clinical frailty in older breast cancer patients, whereas telomere length, IGF-1 and MCP-1 only correlated with age. Plasma IL-6 should be further explored as frailty biomarker in cancer patients.

INTRODUCTION

Chronological age does not always accurately reflect functional status and life expectancy. Frail patients exhibit severely reduced physiological reserves that render them susceptible to minor stressors. More and more oncological therapies become available to older cancer patients, but an accurate evaluation of clinical frailty and general health status is crucial in treatment decision making. Geriatric assessment (GA) is currently the gold standard to evaluate the global health status and clinical frailty level of individuals [1] and is feasible in clinical practice [2], but has several drawbacks. Firstly, it is time-consuming and therefore difficult to integrate in routine clinical practice, although the use of screening tools might partly overcome this problem [3]. Secondly, although GA corresponds with important outcome measures like patient survival and toxicity of treatment, its predictive capacity is moderate, and there is certainly room for better tools. Thirdly, as it does not yield a validated 'end score', it is difficult to precisely quantify the patients' global health status. For this reason, some attempts to summarize and categorize GA results have been proposed in geriatric oncology such as the Balducci score, but the included elements and cut-offs are arbitrary, and do not capture the complexity of the entire ageing process (e.g. age $\geq$ 85 is sufficient to be categorized as 'frail', although it has been stated in the geriatric literature that more than half of patients above 85 are actually not frail) [4].

Several biological ageing and frailty markers described in experimental geriatrics have been proposed to reflect 'biological age' more accurately than clinical assessment [5], but this still needs to be proven. In fact, it is not always clear whether these biomarkers merely reflect chronological age, or rather the presence of clinical frailty. Anyhow, these ageing/frailty biomarkers have not yet filled the gap from bench to bedside to date. In addition, it should be noted that the oncogeriatric field represents a specific niche where extrapolation of general findings from geriatrics research may not be fully valid.

Telomere length represents one of the best documented markers of ageing. As telomeres were shown to represent some type of cellular 'mitotic clock', mean leukocyte telomere length is commonly accepted as a promising ageing biomarker. Indeed, progressive telomere attrition with increasing age has been reported repeatedly. Moreover, shorter telomeres have been linked to age-related disorders such as dementia, cardiovascular diseases, osteoporosis, chronic obstructive lung diseases, cancer, and, most importantly, to a significantly higher mortality rate in the elderly [6]. The association between telomere length and frailty or disability is however much less clear from the literature.

Plasma IL-6 levels have also been associated with mortality and/or worse outcome of several diseases, particularly cardiovascular pathology [7-9]. Several investigators have specifically linked circulating IL-6 levels to the frailty syndrome [10-13]. Notably, IL-6 rising is not an isolated phenomenon but must be seen in the context of a general age-related increase in inflammation markers, called "inflammageing" [14]. Similar age-related differences have also been described for certain inflammatory chemokines such as MCP-1 (also named CCL2, CC-chemokine ligand 2) [15-19] , a protein known for its potent ability to attract and activate monocytes/macrophages. Circulating levels of RANTES, another member of the CC-chemokine subfamily, tend to change during ageing [19]. In addition to the above described molecules, certain endocrine markers also show age-related changes. More specifically, IGF-1 is inversely correlated with increasing age [20]. In mice models, disruption of the GH/IGF-1 signaling network resulting in IGF-1 reduction is associated with increase in oxidative stress in the liver, reduced lifespan, and reduced skeletal density [21]. In humans, low IGF-1 levels have been associated with frailty and decreased functionality [22] Although these candidate ageing/frailty biomarkers have been described in the geriatric field, their value in the clinical practice is far from established, and they have not formally been correlated with the frailty syndrome [23] in older cancer patients. The present study was undertaken to validate the five potential ageing/frailty markers mentioned above in a retrospective cohort of older breast cancer patients. In particular, we wanted to investigate the relationship between these markers and calendar age on the one hand, and with the different components of standard geriatric assessment on the other hand.

RESULTS

Patient and tumor characteristics of included subjects

In total, 244 patients were included in the analysis, of which 82 and 162 were assigned to the young and older patient groups, respectively. Median ages in the young and older groups were 40.0 years (range 27-56) and 76.0 years (range 70-90), respectively. Clinical tumor characteristics are displayed in Table 1. Descriptive statistics of all geriatric test items performed on patients from the older cohort (screening tools and geriatric assessment) are summarized in Table 2. Dependency at ADL was noted for 49.4% of patients, and 53.9% of patients showed dependency at iADL. According to Balducci's criteria, 24.1% of older patients (N=162) scored 'fit', 25.3% 'vulnerable' and 50.6% 'frail'. Our newly developed LOFS could be calculated only for patients who completed all the tests contributing to the scoring (N=130), and the median score was 8 (Q1= 7, Q3= 9).

Correlation of ageing biomarkers with calendar age

Four of the measured biomarkers showed significant association with calendar age (Figure 1). The strongest association was found for circulating IGF-1 levels (Fig. 1A). The plasma biomarkers IL-6 and MCP-1 also showed significant age-related changes, whereas no significant relationship with age could be demonstrated for RANTES. Mean leukocyte telomere length, measured as T/S ratio (which could be measured in 76 young and 120 old patients), significantly decreased with increasing age.

Relation between ageing biomarkers and clinical markers of frailty

First, we correlated the different biomarkers with frailty level according to the Balducci score. We found no difference in mean leukocyte telomere length between 'fit', 'vulnerable' and 'frail' patients (N=120), median T/S ratios being 0.6, 0.7 and 0.7, respectively ($p = 0.391$). Likewise, IGF-1, RANTES and MCP-1 plasma levels did not show any correlation with Balducci category (all $p>0.4$). In contrast, IL-6 plasma levels

were significantly different between the 3 Balducci categories (N= 158): median values for fit, vulnerable and frail subjects were 1.4, 2.3 and 2.8 pg/ml, respectively (p = 0.019). Box plots are shown in Figure 2.

Table 1. Summary of clinical characteristics of young and old patients.

Clinical characteristics	Young patients	Older patients
	N=82	N=162
Age (median in years, [IQR])	40.0 [37.0 - 44.0]	76.0 [72.0 - 80.0]
BMI (median, [IQR])	23.0 [21.3 - 25.6]	26.5 [23.9 – 29.8]
Neoadjuvant hormonal treatment (N/total, %)	8/82 (9.8%)	18/162 (11.1%)
Grade (%)		
I	14.6	15.4
II	47.6	47.5
III	37.8	36.4
unknown	0	0.6
pT[a] (N/total, %)		
1	35/74 (47.3%)	55/144 (28.1%)
2	33/74 (44.6%)	79/144 (54.9%)
3	4/74 (5.4%)	8/144 (5.6%)
4	1/74 (1.4%)	2/144 (1.4%)
x	1/74 (1.4%)	0/144 (0%)
pN[a] (N/total, %)		
0	44/74 (59.5%)	83/144 (57.6%)
1	15/74 (20.3%)	42/144 (29.2%)
2	9/74 (12.2%)	10/144 (6.9%)
3	6/74 (8.1%)	8/144 (5.6%)
x	0/74 (0%)	1/144 (0.7%)
Histological subtype (N/total, %)		
ductal	74/82 (90.2%)	111/162 (68.5%)
lobular	8/82 (9.8%)	25/162 (15.4%)
ductal + lobular	0/82 (0%)	2/162 (1.2%)
ductal + other	0/82 (0%)	5/162 (3.1%)
other	0/82 (0%)	19/162 (11.7%)
'Molecular' subtype (N/total, %)		
Lum A	49/82 (59.8%)	99/162 (61.1%)
Lum B	15/82 (18.3%)	29/162 (17.9%)
Lum B - Her2	6/82 (7.3%)	12/162 (7.4%)
Her2	4/82 (4.9%)	8/162 (4.9%)
Triple Neg	8/82 (9.8%)	14/162 (8.6%)

[a] Only for patients who received upfront surgery

Abbreviations: N= number of patients, IQR= interquartile range, BMI = body mass index, pT = pathological tumor size, pN = pathological lymphnodal status, Lum A= luminal A, Lum B= Luminal B, Her2= Human epidermal growth factor receptor 2 positive tumor, Triple Neg= triple negative tumor.

Table 2. Descriptive statistics of all geriatric test items in the older cohort.

	N	%	95% CI
ECOG-PS (0–5)	153		
Score 0 = asymptomatic	85	55.6	47.5 – 63.6
Score 1 = symptomatic but completely ambulatory	45	29.4	22.0 – 36.8
Score 2 = symptomatic, <50% in bed during the day	4	2.6	0.02 – 5.2
Score 3 = symptomatic, >50% in bed, but not bedbound	17	11.1	6.0 – 16.2
Score 4 = bedbound	2	1.3	0 – 3.1
fTRST	161		
Absence of a geriatric risk profile: score 0	41	25.5	18.6 – 32.3
Presence of a geriatric risk profile: score ≥ 1	120	74.5	67.7 – 81.4
G8 (0–17)	141		
Absence of a geriatric profile: score >14	62	44	35.7 – 52.3
Presence of a geriatric profile: score ≤14	79	56	47.7 – 64.4
ADL (6–24)	162		
Independent: score 6	82	50.6	42.8 –58. 5
Dependent: score ≥ 7	80	49.4	41.5 – 57.2
iADL (0–8)	142		
Completely independent: score 8	67	47.18	38.8 – 55.6
Dependent: score <8	75	52.82	44.4 – 61.2
MMSE (0–30)	156		
Score ≥24 = normal cognition	142	91	86.4 – 95.6
Score 18–23 = mild cognitive decline	13	0.3	0 – 1.2
Score ≤17 = severe cognitive decline	1	0.6	0 – 1.9
GDS-15	156		
Score 0-4 = not at risk for depression	134	85.9	80.3 – 91.4
Score 5-15 = at risk for depression	22	14.1	8.5 – 19.7
MNA-SF (0–14)	141		
Normal nutritional status: score ≥12	79	56	47.7 – 64.4
Risk of malnutrition: score ≤11	62	44	35.6 – 52.3
MNA (0–30)	68		
Score ≥24 = normal nutritional status	21	30.9	19.7 – 42.1
Score 17 to 23.5 = risk of malnutrition	45	66.2	54.7 – 77.6
Score <17 = malnutrition	2	2.9	0 – 7.0
CCI (0–37)	162		
No comorbidities (score 0)	93	57.4	49.6 – 65.2
Comorbidity score 1	34	21	14.6 – 27.4
Comorbidity score ≥2	35	21.6	15.1 – 28.1

Table 2. Continue

LOFS	130		
0	0	0	–
1	6	4.6	00.9 – 08.3
2	2	1.5	0 – 03.7
3	2	1.5	0 – 03.7
4	1	0.8	0 – 02.3
5	8	6.2	1.9 – 10.4
6	11	8.5	3.6 – 13.3
7	24	18.5	11.7 – 25.3
8	24	18.5	11.7 – 25.3
9	26	20	13.0 – 27.0
10	26	20	13.0 – 27.0
Balducci score	162		
Fit	39	24.1	17.4 – 30.8
Vulnerable	41	25.3	18.5 – 32.1
Frail	82	50.6	42.8 – 58.5

Abbreviations: N= number of patients; 95%CI = 95% confidence interval. LOFS: Leuven Oncogeriatric Frailty Score; ECOG-PS: Eastern Cooperative Oncology Group Performance status; fTRST: Flemish version of the Triage Risk Screening Tool; ADL: Activities of Daily Living; iADL: instrumental Activities of Daily Living; MMSE: Mini Mental State Evaluation; GDS: Geriatric Depression Scale; MNA: Mini Nutritional Assessment; MNA-SF: MNA-Short Form; CCI: Charlson Comorbidity Index

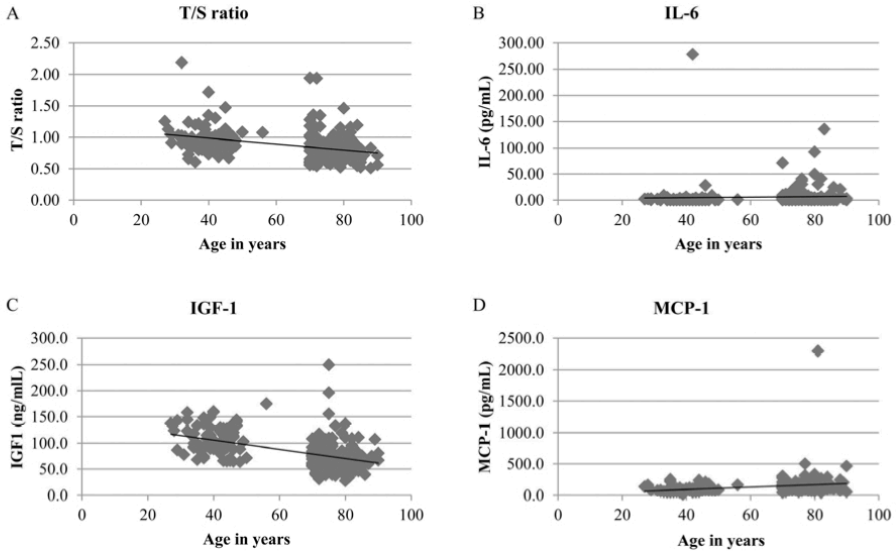

Figure 1. Trends of the 4 biomarkers showing significant association with chronological age. (**A**) Mean telomere length (expressed as T/S ratio) versus age. N=196, Spearman correlation coefficient (r_s) = -0.396, p<0.0001. (**B**) IL-6 versus age. N=238, r_s = 0.272, p<0.0001. (**C**) IGF-1 versus age. N=213, r_s = -0.529, p<0.001. (**D**) MCP-1 versus age. N=238, r_s = 0.412, p<0.0001. For graphical reasons, two outliers are not shown in both the IL-6 and MCP-1 scatterplots: IL-6 of 277.98 pg/ml in a patient aged 42 years, and MCP-1 of 2296 pg/ml in a patient aged 81 years.

Table 3. Spearman correlations between IL-6 and continuous geriatric assessment scores in the old cohort

GA scores	IL-6		
	N	r_s	p
LOFS	129	-0.218	0.0131
ECOG	149	0.244	0.0028
fTRST	157	0.078	0.3288
G8	137	-0.129	0.1320
ADL24	145	0.205	0.0134
IADL8	141	-0.202	0.0163
MMSE	152	-0.093	0.2525
GDS_15	152	0.028	0.7329
MNA-SF	137	-0.118	0.1691
MNA30	65	-0.368	0.0026
CCI	158	0.154	0.0539

Abbreviations: N = number of patients, r_s = Spearman correlation coefficient, p= p value. LOFS: Leuven Oncogeriatric Frailty Score; ECOG-PS: Eastern Cooperative Oncology Group Performance status; fTRST: Flemish version of the Triage Risk Screening Tool; G8: /; ADL: Activities of Daily Living; iADL: instrumental Activities of Daily Living; MMSE: Mini Mental State Evaluation; GDS: Geriatric Depression Scale; MNA-SF: Mini Nutritional Assessment-Short Form; CCI: Charlson Comorbidity Index

Next, we studied the relationship between the different biomarkers and LOFS, and made similar observations. No association was found between LOFS and telomere length, IGF-1, RANTES or MCP-1. For IL-6 however, a significant correlation with LOFS was observed (p=0.0131) (Table 3).

Lastly, we evaluated possible correlations of the different biomarkers with all the separate GA items that are evaluated on a semi-continuous scoring scale: ECOG-PS, fTRST, G8, ADL, iADL, MMSE, GDS15, MNA-SF and CCI. Neither telomere length, nor RANTES and MCP-1 correlated with any of the above mentioned items (all p>0.070, data not shown). Circulating levels of IGF-1 were inversely correlated with CCI (N=133; Spearman correlation coefficient= -0.195, p= 0.0248) (Supplementary Table 2) but not with any of the other GA parameters. The most convincing results were obtained for IL-6 (Table 3): there was a significant correlation with ECOG-PS, ADL, iADL, and a borderline significant correlation with the CCI (p=0.0539). IL-6 levels were, however, not associated with MMSE, MNA-SF, nor with any of the geriatric screening tools (fTRST and G8). Furthermore, we

examined if IL-6 levels were associated with the incidence of falls during the past year. Although patients giving a positive answer to this question showed slightly increased IL-6 levels (median IL-6 value 2.7 pg/ml versus 2.3 pg/ml for patients giving a negative response), the difference was not statistically significant (p=0.154). Similarly, none of the other biomarkers correlated with falls.

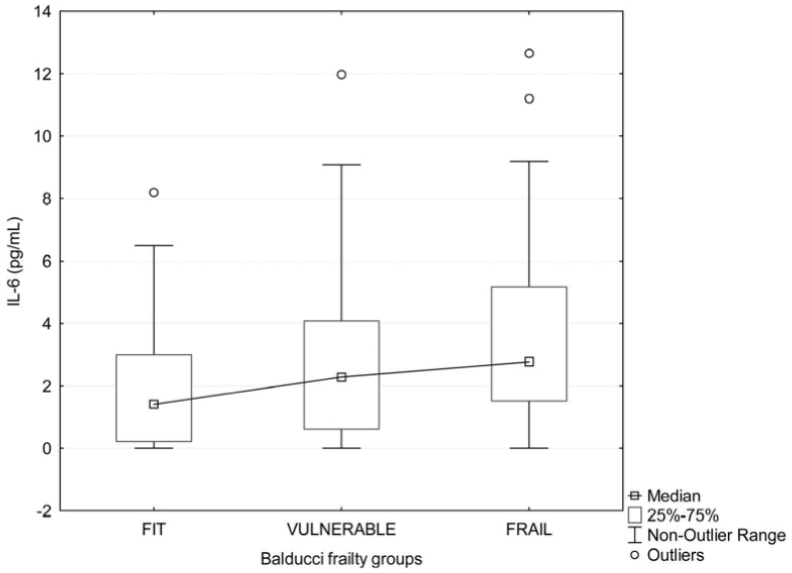

Figure 2. Boxplot showing the relation between plasma IL-6 and frailty status determined by Balducci Frailty Score. Frailty groups according to Balducci's test are displayed on the X axis. In each group, some extreme values are not shown for graphical reasons (2 values in 'fit', 2 values in 'vulnerable', and 8 values in 'frail' group).

Table 4. Correlations between biomarkers in the entire cohort (young and older patients).

Biomarker 1	Biomarker 2	Spearman's correlation coefficient	p-value
Telomere length	IL-6	-0.15553	0.0317
	MCP-1	-0.13009	0.0721
	RANTES	-0.04132	0.5693
	IGF-1	0.25608	0.0006
IL-6	MCP-1	0.22944	0.0004
	RANTES	0.02476	0.7051
	IGF-1	-0.24374	0.0003
MCP-1	RANTES	0.01760	0.7871
	IGF-1	-0.34022	<.0001
RANTES	IGF-1	0.07644	0.2667

Correlations between the different ageing biomarkers

We also evaluated possible relationships between the different ageing biomarkers. Table 4 shows that, except for RANTES, all the biomarkers (telomere length, IL-6, MCP-1, IGF-1) correlated with each other to a lesser or higher extent.

Correlations between ageing biomarkers and tumor characteristics

No significant association was found between any of the ageing biomarkers and tumor size (pT)[29], nodal status (pN), histologic breast carcinoma subtype (ductal, lobular, combined or other) or molecular tumor subtype (luminal A, luminal B/Her2-, luminal B/Her2+, non-luminal Her2+, triple-negative) (data not shown).

DISCUSSION

In this study we have investigated the associations between calendar age and clinical ageing/frailty on the one hand, and a panel of biological markers, measurable in blood, on the other hand. In addition, we also describe a novel approach to compile the results of the different geriatric assessment test items into a single output score on a 10-points scale.

Geriatric assessment is currently the best way to assess the level of fitness in oncogeriatric patients, in order to plan an adequate therapeutic strategy. It allows to detect unknown geriatric problems and to adapt treatment regimens accordingly [2, 30-32]. GA consists of different questionnaires and tests that have been validated independently, each reflecting a specific aspect of the geriatric phenotype. However, interpreting GA results and translating them into a risk profile is challenging, since no real 'end score' exists. Several frailty models have been developed in general geriatrics, but Balducci and Extermann were the first to suggest a classification of cancer patients into 3 groups ('fit', 'vulnerable' and 'frail') depending on their GA result [33], with the purpose of guiding treatment decisions and predict life expectancy. For instance, frail patients would solely be administered palliative treatments, while a specific individualized approach (e.g. dose reduction at the start with subsequent escalation) would be applied for vulnerable patients. Considering the complexity of ageing, and the wide variety of aspects that are evaluated by GA, the Balducci classification is a simplified tool with many shortcomings and was probably meant as a starting point for future refinement. We have developed a new method, designated LOFS, to calculate a global GA end score that integrates 5 fundamental aspects determining a patient's fitness/frailty status, i.e. capability to autonomously perform activities of daily living (ADL and iADL), mental state (MMSE), nutritional state (MNA-SF) and comorbidities (CCI). A direct comparison between Balducci score and LOFS was not possible, as there is no gold standard to compare them to. However, LOFS is more refined than the Balducci score, as its semi-continuous scoring system is in line with recent evolution in the geriatric research field, where frailty is more and more seen as a continuous event (cumulative deficit model) [4]. In keeping with the Balducci criteria, a patient can be categorized as 'frail' solely based on advanced age (85 years or older), or sporadic incontinence. However, patients fulfilling these criteria are not necessarily frail individuals with a global health status being too poor to tolerate cancer chemotherapy. Undertreatment, due to fear of toxicity or intolerance, is a frequent problem in older breast cancer patients and results in an increased risk of relapse [34]. The value of LOFS in predicting toxicity of treatment and general outcome (survival) of patients will be comprehensively assessed in future studies (we are currently conducting a large survival study in a separate cohort).

As GA alone is not sufficient to predict outcome and tolerance to chemotherapy[35, 36], there is an increasing interest to integrate it with specific biomarkers that reflect biological ageing and frailty in individuals affected by cancer, in order to get a more complete picture of the patients' physiological reserves and capability to tolerate chemotherapy. Despite the well-documented correlation of several measurable molecules with age/frailty in the general (non-cancer) population [9-13, 15, 17, 18, 20, 22], none of these markers has emerged as "the" ageing biomarker of choice. Even less information is available on ageing biomarkers that could be valuable in oncogeriatric patients. This is partly due to complexity arising from the extent of the malignant disease, mechanisms of ageing and possible interactions between these two processes. We have explored several candidate ageing biomarkers in our breast cancer study cohort, and examined their relation to the patient's frailty status, either according to Balducci or our newly developed LOFS. Our results show that the biomarkers correlate at least as good with age/frailty defined according to LOFS, as Balducci. In our patient population, plasma IL-6 emerged as the strongest frailty marker. It was not only associated with Balducci category and LOFS, but also with other items of the GA not included in the calculation of these global scores, like for instance ECOG-PS. A correlation between plasma IL-6, ageing/frailty and even with mortality had previously been consistently observed in numerous studies on

general (non-cancer) geriatric populations [7-13, 37]. We have shown that plasma IL-6 also correlates with calendar age. Age-related rising of circulating IL-6 is believed to originate from ageing of the immune system, generally referred to as 'immunosenescence'. This results in an altered profile of circulating leukocytes, but also provokes an imbalance of pro- and anti-inflammatory cytokines and chemokines. This ageing-related pro-inflammatory status, called 'inflammageing', has been correlated with dementia, Parkinson's disease, atherosclerosis, type 2 diabetes, sarcopenia, functional disability and death [14, 38]. We now demonstrated that circulating IL-6 levels are associated with both chronological age and frailty in a selected breast cancer population. The additional predictive value of IL-6, supplementary to that of clinical frailty assessment, for outcome and treatment toxicity in (breast) cancer patients should be explored in the future.

For plasma MCP-1 we found a strong correlation with calendar age but no association with clinical frailty. Although association of MCP-1 with atherosclerosis has been documented, circulating MCP-1 levels seem to be primarily correlated with chronological age [15-19]. In line with these earlier reports, our results suggest that MCP-1 merely reflects chronological age, which does not always accurately mirror the patient's physiological reserves and functional status. The lack of association with clinical frailty does not make MCP-1 an attractive biomarker to be used in the clinic for treatment decisions. Data on the chemokine RANTES/CCL5 are even less consistent: RANTES has been proposed as an ageing biomarker because its plasma concentrations have been shown to increase with ageing [39], but in our study we did not find any association with calendar age nor with clinical frailty.

As expected, mean leukocyte telomere length inversely correlated with chronological age in our study population. However, we did not detect any correlation between mean telomere length in circulating white blood cells and functional status. Shorter telomeres have been linked to age related diseases, a higher mortality rate [6], and also to premature ageing syndromes like progeria or trichotiodystrophy [40, 41]. However, studies investigating the relationship between telomere length and frailty/disability showed conflicting results [42-48]. It has been suggested that biomarker associations with health outcomes may differ between very old and younger old populations (through a positive selection effect or survivor effect) [6, 45, 49]. Our population did not contain an overload of 'very old', as the median age was 76 years and only 16% of patients were above 80. Nevertheless, we did not find a significant relationship between telomeres and frailty. It should be kept in mind, though, that the oncogeriatric population is probably a biased one, as severely frail older cancer patients will rarely be referred to the oncologists for specialist care. In this regard, observations made on general geriatric populations might not be valid in oncogeriatric populations.

To our knowledge, the association between functional status and mean leukocyte telomere length has never been investigated in older cancer patients; our study is the first to report the lack of such correlation in this specific setting.

The relevance of the IGF-1 pathway in mammalian longevity was initially demonstrated in rodents (calorie restriction was shown to decrease IGF-1 levels with increased lifespan as result) and knockout mice [50]. In humans, low IGF-1 levels have been shown to inversely correlate with increasing age [20], and some studies suggest a link with frailty/functionality [22, 51]. In our study population, IGF-1 showed no association with GA components, except for a significant correlation with CCI. Yet, compared to the other investigated molecules, IGF-1 showed the strongest association with calendar age. Thus, like plasma MCP-1, circulating IGF-1 seems to be linked with the chronological age but not with ageing-related functional decline and frailty.

In conclusion, we have investigated the relationship between several potential biomarkers of ageing/frailty, and the different components of the GA, within a specific breast cancer population. We confirmed IL-6 as a promising marker in predicting frailty, besides its correlation with age. MCP-1, IGF-1 and leukocyte telomere length were correlated with chronological age, but not with frailty, and apparently reflect only the time aspect of ageing, but not the possible functional consequences (i.e. clinical frailty) of the ageing process. However, lack of correlation with frailty status at the time of diagnosis does not necessarily mean that these markers have no value in guiding treatment choices. Hence, the next steps to be undertaken are prospective validation of these and perhaps other markers like for instance circulating microRNAs, in predicting outcome including survival and short- and long term toxicity from different treatment modalities. We are currently conducting an extensive prospective ageing biomarker study in breast cancer patients receiving chemotherapy. The ultimate goal would be the identification of robust frailty biomarkers that can add on, or maybe even (partly) replace, the extensive clinical GA that is suggested nowadays.

We also designed the LOFS, a novel comprehensive method to categorize patients on the basis of their GA

results. This new 10-point scoring system reflects the general condition of a patient in a more refined way and allows a more subtle interpretation of the GA results taking into account 5 crucial domains of GA, while at the same time retaining the simplicity of a single end score that is desirable for application in daily practice.

The field of medicine in general, but especially geriatric oncology, is evolving more and more towards a patient-tailored approach in which accurate frailty assessment instruments, such as easy-to-measure biomarkers and reliable but simple clinical evaluation tools, could be very helpful.

MATERIALS AND METHODS

Patient selection

1. Selection of the older patients group. From 2004 on, several prospective projects integrating geriatric assessment in older cancer patients were performed in our center (University Hospitals Leuven, Leuven, Belgium) and all results obtained throughout the years were gathered in a GA database. From this database, all patients aged ≥ 70 years with new diagnosis of early or locally advanced (i.e. non-metastatic), primary or second primary breast cancer with GA performed before initiation of any chemotherapy, radiotherapy or surgery, were retrospectively selected. Neo-adjuvant antihormonal treatment was allowed between the time of diagnosis and the time of GA, since we expected virtually no, or only very minor impact on the result of GA. From this primary selection, we chose patients for whom a blood sample collected at diagnosis (i.e. before administration of any treatment or performance of any surgical procedure) was available from the large-scale breast cancer blood bank that was established at our hospital from 2003 onwards by the Leuven Multidisciplinary Breast Center (LMBC), and that contains blood specimens from more than 4000 breast cancer patients. In total, 162 patients fulfilled all these inclusion criteria.

2. Selection of the young patients group. A second group, consisting of younger breast cancer patients, was selected from the LMBC database and biobank. Inclusion was based on the same criteria as described above, except for age and GA. We aimed for a broad age range below the cut-off of 60 years. From this group, a final selection of 82 patients was made so as to ensure that both cohorts (older versus young) contained similar percentages of Luminal A-like, Luminal B-like, Luminal B-HER2 positive, HER2 positive and Triple Negative breast tumors, as defined in one of our previous publications [24].

Collection of plasma and leukocyte DNA. Blood sampling and isolation of plasma (collection started in 2003) and DNA (collection started in 2007) for experimental use is routinely performed in our hospital, in all new breast cancer patients who give written informed consent for the LMBC biobanking project. The procedures were previously described by Hatse S. et al. [25].

Measurements of cytokine/chemokine levels in plasma. IL-6 was measured using LEGEND MAX™ ELISA kit (BioLegend). The analysis for CCL5/RANTES, CCL2/MCP-1, and IGF-1 was carried out with Quantikine ELISA kit (R&D Systems). All procedures were performed following the manufacturers' instructions. Read-out was performed by dual spectrophotometric measurement: absorbance measured at 570 nm was subtracted from absorbance measured at 450 nm. All samples were assayed in duplicate. On each microplate, a standard curve, obtained from dilution of a standard with known concentration, was included. Concentrations of samples were calculated from the standard curve using a logistic curve-fitting algorithm.

Mean leukocyte telomere length. Mean leukocyte telomere length was measured for all patients with a leukocyte DNA sample collected at diagnosis (i.e. patients diagnosed in 2007 or later). Every DNA sample was first tested for DNA fragmentation by electrophoresis on a 1% agarose gel. Fragmented DNA samples were excluded from further analysis. Telomere length was determined using the qPCR-based method developed by R. Cawthon [26]. Briefly, the relative amount of telomeric DNA ("T/S ratio") is calculated based on the Cp values obtained for telomeric DNA ("T") and for the single-copy housekeeping gene 36B4 ("S"), measured in the same sample. All samples were assayed twice in independent qPCR runs, each time in triplicate wells. Each run included a dilution series (i.e. 80, 20, 5 and 1.25 ng) of human standard DNA (Human Genomic DNA, Roche). The "T/S ratio" for an experimental sample is the amount (ng) of standard DNA that matches the experimental sample for copy number of the telomere template ("T"), divided by the amount (ng) of standard DNA that matches the experimental sample for copy number of the single-copy gene ("S") Primer pairs used were 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3' and 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3' for telomeres and 5'-CAGCAAGTGGGAAGGTGTAATCC-3' and 5'-CCCATTCTATCATCAACGGGTACAA-3' for 36B4. The reaction mixture contained 1x LightCycler 480 SYBR Green I Master (Roche, Indianapolis, US), telomere or 36B4 forward and reverse primers at 0.6

µM (telomeres)or 0.5 µM (36B4) each, and 20 ng of template DNA in a total volume of 20 µL. Plates were run on a Roche LightCycler 480 platform, using the following thermal cycling program : activation for 10 min at 95°C; two initiation cycles of 15s at 95°C followed by 15s at 49°C; 35 amplification cycles of 15s at 95°C, 10s at 60°C and 15s at 72°C. Thereafter, melting curves were also established to check amplicon purity.

Geriatric assessment. Patient scores at different tests, included in the GA, were available from our GA database. The following items were mostly available: the screening tools G8 and Flemish version of the Triage Risk Screening Tool (fTRST), Eastern Cooperative Oncology Group Performance Status (ECOG-PS), functional status measured by Activities of Daily Living (ADL) and instrumental Activities of Daily Living (iADL), Geriatric Depression Scale (GDS-15), Mini Mental Evaluation-30, and Mini Nutritional Assessment- 14 items (MNA-SF). Since the geriatric assessments performed in the different oncogeriatric projects constituting our GA database were not exactly identical, there were rare missing cases for some of the scales, but for the majority, all scale results were available. Charlson Comorbidity Index (CCI) at the time of diagnosis was calculated retrospectively for each patient using the electronic patient files. More details on the scales and references can be found in another recently published paper from our group [2].

Balducci score. A level of frailty was assigned to each patient using the criteria suggested by Balducci and Extermann [27, 28]. 'fit' was assigned to patients without ADL or iADL impairment (i.e. patients independent at all items), and with no or only mild comorbidities; 'vulnerable' was assigned to patients with dependency at 1 or more iADL items, and/or with 1 or 2 severe comorbidities; 'frail' was assigned to patients ≥85 years of age, or patients dependent at 1 or more ADL items, and/or exhibiting 3 or more severe comorbidities. Patients with documentation of one or more geriatric syndromes (dementia, falls, delirium, depression, incontinence, osteoporosis, neglect and abuse, failure to thrive) were also categorized as 'frail'.

Leuven Oncogeriatric Frailty Score (LOFS). In the geriatrics world, frailty is more and more seen as a cumulative deficit disorder, and should thus be appraised as a continuous spectrum, rather than a dichotomized or trichotomized status[4]. Therefore, we developed the LOFS, a semi-continuous frailty score, based on internationally validated cut-offs for ADL, iADL, MMSE, MNA-SF and CCI. A detailed overview of the score compilation is shown in Supplementary table 1. The scoring range for each separate test is trichotomized, the lowest part (worst score range for this particular test) resulting in a LOFS +0 (no contribution to the final 10-points LOFS score), the middle part in +1 (contribution of 1 point to the final score), and the highest part in +2 (contribution of 2 points). Contributions from the 5 tests are added up to result in a total score on a scale from 0 (poorest score; patient suffering from extreme frailty) to 10 (best score, fit patient). Individual results from the LOFS should be interpreted as a gradation of severity in the spectrum of frailty between both extremes.

Statistical analysis. We analyzed correlations between ageing biomarkers and calendar age, and between ageing biomarkers and clinical frailty (defined as by Balducci test or LOFS). In addition, we also studied correlations of ageing biomarkers with each of the individual geriatric assessment tools separately and correlations among the different ageing biomarkers. Since distinct breast tumor subtypes might have a different impact on the host and tumor stroma (e.g. triple negative subtype are associated with stronger immune response), we also studied the influence of tumor subtype, tumor stage (pT) and nodal status (pN) on the biomarkers.

Associations between continuous and discrete variables were evaluated using the Mann-Whitney U test (for two levels) or the Kruskal-Wallis test (for more than two levels). Associations between two continuous variables, or between a continuous and an ordinal categorical variable, were analyzed by the Spearman correlation coefficient. Associations between two discrete variables were analyzed using the Fisher exact test.

The cut-off for statistical significance was set at $p = 0.05$.

Ethical aspects. The LMBC biobank project and this study have been approved by the Ethics Committee of the University Hospitals Leuven.

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Conflict of interest statement

None of the authors have potential conflicts of interest to disclose.

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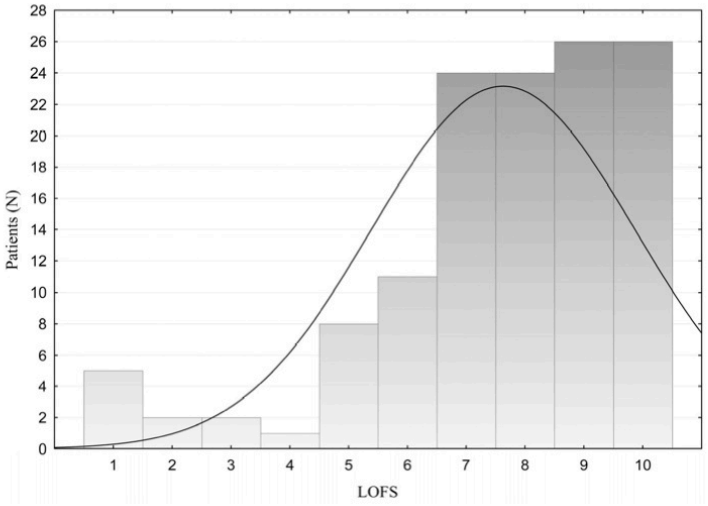
SUPPLEMENTARY INFORMATION

Supplementary Table 1. Leuven Oncogeriatric Frailty score

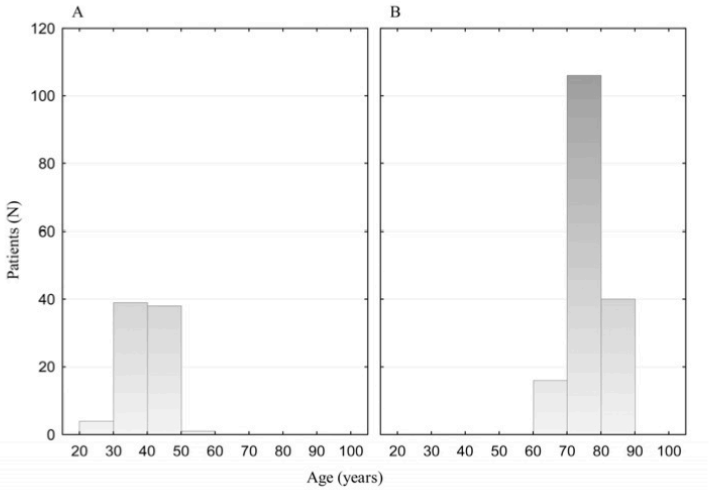
Geriatric test	LOFS +2	LOFS +1	LOFS +0
ADL	6	5 - 4	≤ 3
iADL	8	7 - 4	≤ 3
MMSE	30 - 28	27 - 24	≤ 23
MNA	14 - 12	11 - 8	≤ 7
CCI	0	1	≥ 2

Supplementary Table 2. Spearman correlations between biomarkers and continuous geriatric assessment scores in the old cohort

GA scores	Telomeres			IGF-1			MCP-1			RANTES		
	N	r_s	p	N	r_s	p	N	r_s	p	N	r_s	p
LOFS	109	0.048	0.6174	114	0.124	0.1897	130	-0.017	0.8463	130	-0.025	0.7799
ECOG	120	-0.068	0.4616	127	-0.094	0.2937	150	-0.026	0.7559	150	0.031	0.7108
TRST	120	0.041	0.6604	132	-0.016	0.8537	157	0.001	0.9927	157	-0.066	0.4111
G8	120	-0.015	0.8740	118	0.041	0.6628	139	-0.112	0.1894	139	0.010	0.9066
ADL24	120	0.092	0.3162	123	-0.139	0.1243	146	0.002	0.9770	146	0.111	0.1815
IADL8	108	0.056	0.5652	123	0.073	0.4211	141	0.085	0.3153	141	0.007	0.9326
MMSE	120	0.088	0.3380	130	0.071	0.4192	153	0.153	0.0596	153	-0.058	0.4781
GDS_15	120	0.131	0.1542	130	-0.026	0.7653	153	-0.146	0.0719	153	0.015	0.8530
MNA14	120	-0.054	0.5559	118	0.096	0.2995	139	-0.053	0.5327	139	0.037	0.6662
MNA30	56	0.040	0.7681	51	0.089	0.5327	66	-0.089	0.4763	66	0.029	0.8162
CCI	120	0.009	0.9182	133	-0.195	0.0248	158	0.123	0.1244	158	-0.055	0.4905



Supplementary Figure 1. LOFS distribution among older patients.



Supplementary Figure 2. Histogram of the age distribution of the study population. (**A**) young patients group (median age = 40 years, IQR = 37 – 44). (**B**) older patients group (median age = 76 years, IQR = 72 – 80).

Results: Chapter 3: The impact of adjuvant chemotherapy in older breast cancer patients on clinical and biological aging parameters.

The impact of adjuvant chemotherapy in older breast cancer patients on clinical and biological aging parameters

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ABSTRACT

Purpose. This prospective observational study aimed to evaluate the impact of adjuvant chemotherapy on biological and clinical markers of aging and frailty.

Methods. Women ≥ 70 years old with early breast cancer were enrolled after surgery and assigned to a chemotherapy (Docetaxel and Cyclophosphamide) group (CTG, *n*=57) or control group (CG, *n*=52) depending on their planned adjuvant treatment. Full geriatric assessment (GA) and Quality of Life (QoL) were evaluated at inclusion (T0), after 3 months (T1) and at 1 year (T2). Blood samples were collected to measure leukocyte telomere length (LTL), levels of interleukin-6 (IL-6) and other circulating markers potentially informative for aging and frailty: Interleukin-10 (IL-10), Tumor Necrosis Factor Alpha (TNF-α), Insulin-like Growth Factor 1 (IGF-1), Monocyte Chemotactic Protein 1 (MCP-1) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES).

Results. LTL decreased significantly but comparably in both groups, whereas IL-6 was unchanged at T2. However, IL-10, TNF-α, IGF-1 and MCP-1 suggested a minor biological aging effect of chemotherapy. Clinical frailty and QoL decreased at T1 in the CTG, but recovered at T2, while remaining stable in the CG.

Conclusion. Chemotherapy (TC) is unlikely to amplify clinical aging or induce frailty at 1 year. Accordingly, there is no impact on the most established aging biomarkers (LTL, IL-6).

INTRODUCTION

The incidence of breast cancer, the most frequent tumor occurring in women, increases with age. While adequate treatment can improve outcome and survival in the elderly, concerns over side effects or the idea of futility result in a lower use of adjuvant chemotherapy in this patient population. This might be one of the reasons why cancer-related mortality is higher in older patients [1]. The high variability of individual health status constitutes a major challenge in offering optimal therapy to the elderly. A comprehensive geriatric assessment (GA), evaluating functional status, comorbidity, socio-economic condition, nutrition and polypharmacy, is therefore necessary, and has been recommended by the International Society of Geriatric Oncology (SIOG) [2]. Based on our own findings, biological markers of aging and frailty could add on to this clinical evaluation [3, 4].

In line with the complexity of the aging process, a huge variety of potential aging biomarkers has been described. A crucial role has been attributed to telomeres, in cells and tissues subjected to replicative aging. They are incompletely replicated in somatic cells and shorten with each cellular division. Therefore, leukocyte telomere length (LTL) can serve as a marker of a cell's replicative "age" [5], and, in extension, can mirror a person's biological age [6]. LTL correlates with several aging-related syndromes [7]. An increasing low-grade chronic inflammatory status, reflected by an altered plasma level of multiple inflammatory mediators [8-10], is another hallmark of aging. Levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) continuously rise with age, and have been associated with several aging-related syndromes [11-13]. Conversely, the anti-inflammatory cytokine interleukin-10 (IL-10) tends to decrease in blood during aging [14] and age-related diseases [15]. Furthermore, several chemokines also change during aging [16-19] : Monocyte Chemotactic protein 1 (MCP-1) blood levels are higher in older people compared to younger individuals [20-22]. Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), has shown to undergo age-related changes as well, although, results from the literature are not consistent [21, 22]. Additionally, perturbation of the insulin/insulin-like growth factor 1 (IGF-1) metabolic pathway has been implicated in aging-related disease, and reduced longevity in both animal models [23-25] and humans [26, 27, 12].

Chemotherapy may influence the aging process via a variety of different mechanisms. Firstly, anticancer agents can induce cellular senescence through DNA damage [28], either directly or indirectly via generation of free radical intermediates and inhibition of DNA repair enzymes. Secondly, chemotherapy may specifically accelerate telomere attrition in leukocytes, most likely due to direct telomere damage or possibly by inhibition of the enzyme telomerase [29]. Repeated cycles of intense hematological repopulation during chemotherapy may shorten telomeres more rapidly if telomerase is not compensating for endochromosomal DNA loss [30-32]. Such effects of anticancer drugs on the replicative capacity of blood cells may be more pronounced in older compared to younger patients [33]. Finally, neuroendocrine and immune functions can also be affected by chemotherapy and by corticosteroids that are often incorporated in chemotherapeutic regimens [34]. Chemotherapy might thus be expected to accelerate aging [35, 36, 33, 37, 38]. It has been hypothesized that an increased rate of molecular aging might explain some of the delayed adverse events linked to chemotherapy [39]. However, long-term follow-up data, on both clinical and biological repercussions of chemotherapeutic treatments, have never been reported

To ensure optimal treatment decisions in older patients, it is of utmost importance to further elucidate the impact of chemotherapy on the aging process, not only biologically, but most particularly in terms of clinical repercussion. Here, we report a prospective study to assess the effect of chemotherapy on biological and clinical aging markers in older patients with breast cancer.

RESULTS

In total, 109 consecutive subjects were enrolled in the study: 57 in the chemotherapy group (CTG) and 52 in the control group (CG). Almost all CTG patients completed their adjuvant chemotherapy. One patient stopped after the first cycle, one after the second cycle and two patients after the third cycle because of adverse events (allergy, severe infection and overall intolerance). Two other patients stopped after 1 cycle because of an allergic reaction, but resumed chemotherapy with a taxane-free, anthracyclin containing regimen. Baseline tumor and treatment characteristics are described in Table 1.

Results of the different biomarker assays at the 3 time points (T0, inclusion; T1, at 3 months; T2, at 1 year) and their evolution over time are shown in Table 2 and Figure 1. In brief, LTL was similar in both cohorts at inclusion, and decreased to the same extent in both groups, indicating no difference in evolution in the two cohorts (test for interaction p=0.88). Also for RANTES, the evolution was similar in both groups. In contrast, the other 5 biomarkers remained stable in the CG while significantly changing in the CTG. IL-6 decreased at T1 and returned to initial levels at T2; MCP-1 decreased at T1 but increased above baseline value at T2; IGF-1 showed a similar initial decline at T1 but only slightly recovered at T2. On the other hand, IL-10 increased at T1 but decreased at T2 and TNF-α levels were increased at both T1 and T2. To determine if differences in baseline frailty between groups could have influenced these results, we repeated the time interaction analysis correcting for frailty at T0. This analysis showed similar results (Table 2).

For background on geriatric assessment and our

Table 1: Baseline patient and tumor characteristics

	Chemo Group (*n* = 57)	**Control Group (*n* = 52)**
Age Median, years (range)	 73.5 (70-80)	 75.0 (70-90)
pT 1 2 3 4	n (%) 11 (19) 37 (65) 6 (11) 3 (5)	n (%) 21 (40) 30 (58) 0 (0) 1 (2)
pN 0 1-3	n (%) 18 (33) 36 (67)	n (%) 27 (53) 24 (47)
Breast cancer phenotype§ Basal like HER2 positive (ER negative) Luminal A Luminal B HER2 negative Luminal B HER2 positive	n (%) 11 (19) 6 (10) 9 (16) 22 (39) 9 (16)	n (%) 0 (0) 0 (0) 35 (67) 16 (31) 1 (2)
Adjuvant therapy TC chemotherapy G-CSF primary prophylaxis Trastuzumab 1 year Endocrine therapy Radiotherapy	n (%) 56 (100) 48 (86) 15 (27) 40 (71) 46 (82)	n (%) 0 (0) 0 (0) 0 (0) 52 (100) 32 (62)

Abbreviations : ER : Estrogen Receptor; TC : Docetaxel-Cyclophosphamide; G-CSF : Granulocyte-Colony Stimulating Factor
§: Breast cancer phenotype : see ref 47 in manuscript, Goldhirsh et al.

newly developed frailty score the 'Leuven Oncogeriatric Frailty Score (LOFS)', we refer to the section patients and methods and appendix 1. GA results at the 3 time points, and the differential evolution over time (with and without correction for frailty) are displayed in Table 3 and Figure 2. A significant interaction test, pointing to a differential evolution in time between both groups, was found for LOFS, instrumental activities of daily living (iADL), Mini Nutritrional Assessment – short form (MNA-SF) and Global Quality of Life (Global QoL), while this test was not significant for Activities of Daily Living (ADL), Mini Mental Status Evaluation (MMSE), Geriatric Depression Scale - 15 (GDS-15) and Charlson Comorbidity Index (CCI). A marked decline in LOFS, iADL, MNA-SF and Global QoL was noted at T1 in the CTG but not CG. However, all significant differences noted at T1 in the CTG returned to normal at T2. No significant modifications of frailty level according to Balducci were found in either of the two groups: the odds ratio for being fit rather than vulnerable, or vulnerable rather than frail according to this index was 0.90 (95% CI 0.27-3.07) from T0 to T1 and 0.63 (95% CI 0.21-1.90) from T0 to T2 in the CTG, and there was no difference with the CG (test for interaction p=0.63) (see Figure 2A).

Within the CTG we explored the influence of baseline frailty on the time evolution of biological and clinical aging markers. Because the very small number of truly frail patients in this chemotherapy group, we chose to dichotomise the patients comparing fit patients to vulnerable+frail patients according to Balducci, and patients with LOFS $\geq$ 8 to patients with LOFS < 8). Except for LTL evolution, that showed a significant time interaction with frailty status (p=0.04 for Balducci dichotomization and p=0.01 for LOFS dichotomization), no differences in evolution according to frailty status at the start were seen for other biomarkers. As for the clinical aging parameters, the evolution over time according to baseline frailty status showed to be different for MNA and Global Health (significant time interaction with Balducci category; p=0.02 and p=0.01 respectively) and for GDS and Falls (significant time interaction with LOFS category; p=0.04 and p=0.01 respectively), but not for CCI, ADL, iADL and MMSE.

Correlations of baseline (T0) aging biomarkers with chronological age and LOFS are shown in Table 4. LTL showed a significant correlation with LOFS but not with chronological age. Of all biomarkers, IL-6 was most strongly associated with both chronological age and LOFS: the higher IL-6, the higher chronological age and the lower the LOFS. TNFα showed a strong and highly significant positive correlation with chronological age. Associations with other aging biomarkers were not significant.

Adverse events occurring during the study period were recorded at 3 months and at one year, and are summarized in Table 5. As expected, toxicity was

Table 2: Aging biomarker results at baseline (T0), 3 months (T1), and 1 year (T2), and their differential evolution over time in Chemo and Control Groups

	Chemo Group (n=57)			Evolution Over Time Chemo Group		Control Group (n=52)			Evolution Over Time Control Group		Differential Evolution Chemo and Control (TimeInteraction)
	T0	T1	T2	T0→T1	T0→T2	T0	T1	T2	T0→T1	T0→T2	
LTL N T/S mean +/- SD	45 0.7 +/- 0.2	46 0.7 +/- 0.3	49 0.6 +/- 0.2	**p=0.05** ***p*=0.05**	**p<0.01** ***p*<0.01**	41 0.7 +/-0.3	45 0.6 +/-0.15	44 0.6 +/-0.14	**p=0.02** ***p*=0.02**	**p<0.01** ***p*<0.01**	p=0.88 *p*=0.87
IL-6 N pg/mL mean +/- SD	56 3.2 +/- 3.7	55 2.3 +/- 3.7	51 4.5 +/- 9.2	**p=0.02** ***p*=0.02**	p=0.27 ***p*=0.26**	52 7.0 +/-13.9	48 11.4 +/-38.5	46 5.6 +/-6.1	p=0.95 *p*=0.77	p=0.66 *p*=0.45	**p<0.01** ***p*<0.04**
IL-10 N pg/mL mean +/- SD	51 0.3+/- 0.4	50 0.3 +/- 0.3	47 0.2 +/- 0.1	**p=0.05** ***p*=0.01**	**p<0.01** ***p*<0.01**	50 0.2 +/-0.2	47 0.2 +/-0.2	46 0.2 +/-0.1	p=0.92 *p*=0.96	p=0.28 *p*=0.23	**p<0.01** ***p*<0.01**
TNF-alpha N pg/mL mean +/- SD	56 2.5 +/- 10.1	55 2.9 +/- 9.7	51 3.3 +/- 9.6	**p<0.01** ***p*<0.01**	**p<0.01** ***p*<0.01**	52 2.3 +/-3.1	48 2.5 +/-3.4	46 2.5 +/-3.1	p=0.71 *p*=0.71	p=0.08 *p*=0.06	**p<0.01** ***p*=0.01**
MCP-1 N pg/mL mean +/- SD	55 143 +/- 70	55 110.7 +/- 70	51 183.2+/- 48	**p<0.01** ***p*<0.01**	**p<0.01** ***p*<0.01**	52 189 +/-78	48 219 +/-131	46 207 +/-108	p=0.14 *p*=0.16	p=0.34 *p*=0.29	**p<0.01** ***p*<0.01**
Rantes N pg/mL mean +/- SD	55 59562+/- 46691	55 61411+/- 53735	51 51903+/- 47600	p=0.78 *p*=0.83	**p=0.01** ***p*=0.01**	52 59004 +/-43436	48 53215 +/-46461	46 55421 +/-48231	**p=0.03** ***p*=0.03**	**p=0.03** *p*=0.03	p=0.29 *p*=0.28
IGF-1 N ng/mL mean +/- SD	55 79 +/- 26	54 67 +/- 26	51 70 +/- 24	**p<0.01** ***p*<0.01**	**p<0.01** ***p*<0.01**	51 76 +/-36	48 77 +/-27	46 74 +/-34	p=0.31 *p*=0.35	p=0.48 *p*=0.64	**p<0.01** ***p*<0.01**

Abbreviations. SD: Standard Deviation; CI: Confidence Interval; LTL: Leukocyte Telomere Length; IL-6: Interleukin-6; IL-10: Interleukin-10; TNF-alpha: Tumor Necrosis Factor Alpha; MCP-1: Monocyte Chemotactic Protein 1; RANTES: Regulated Upon Activation, Normal T cell Expressed and presumably Secreted; IGF-1: Insulin Like Growth Factor 1
p-values in italic font show results corrected for patient frailty level at baseline

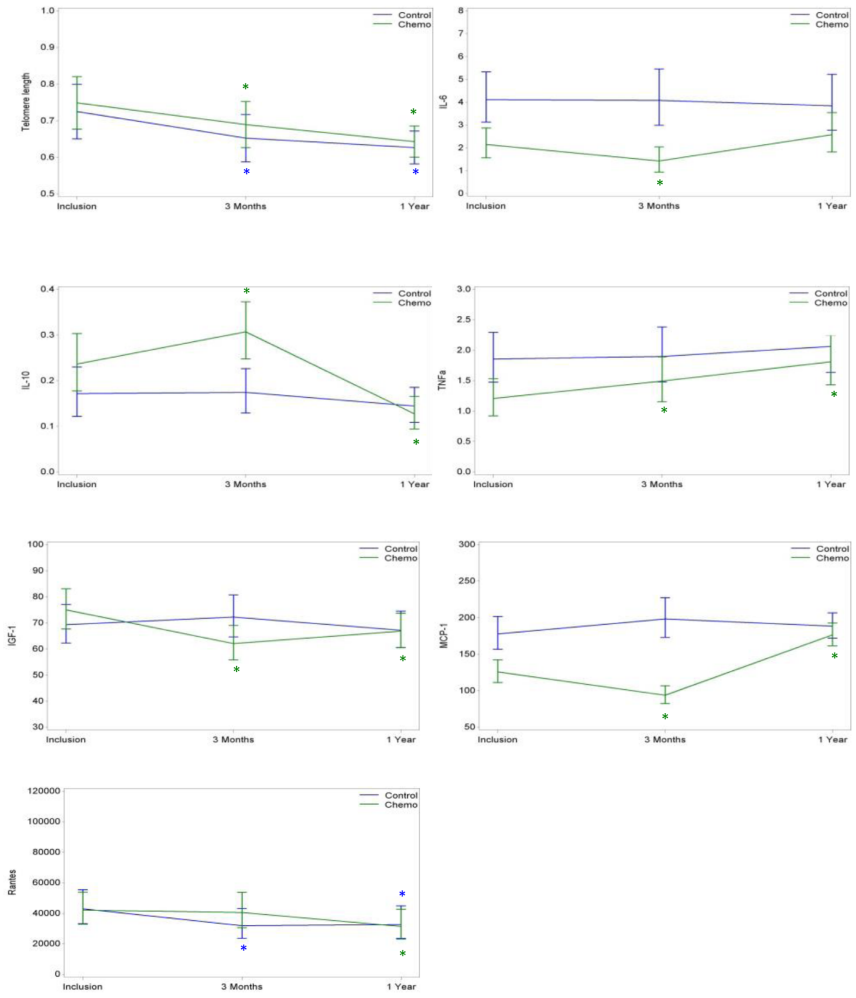
markedly more frequent in the CTG, particularly during treatment (i.e. between T0 and T1). We also assessed whether any of the aging biomarkers could predict the occurrence of grade II-III-IV toxicity at 3 months in the CTG. Analyses were performed for toxicity parameters that occurred in at least 5 patients. None of the aging biomarkers at baseline (T0) predicted development of grade II or higher toxicity, and neither did the Balducci score or LOFS (data not shown).

Unplanned readmissions occurred between 0 and 3 months in 12 patients (22%) of the CTG (N=54) and 3 patients (6%) of the CG (N=50). Between 3 and 12 months, these numbers were 9 (18%) for the CTG and 15 (32%) for the CG. However, none of the biomarkers at baseline (T0) nor Balducci or LOFS predicted an unplanned readmission during chemotherapy.

DISCUSSION

Geriatric oncology is a growing discipline. Older breast cancer patients have a higher cancer-specific mortality [1], probably because therapy is withheld on concerns over side effects. Is this fear justified and are these suspected side effects actually related to the aging process? Some studies seem to show an accelerating effect of chemotherapy on the aging process [33, 35, 36, 38]. One could anticipate an increase in geriatric problems after chemotherapy. This could mislead the oncologist not to administer chemotherapy where it would otherwise have been indicated. However, data on this topic still remain disparate to date. DNA damage (and DNA damaging drugs) are suggested not necessarily to cause or accelerate aging [40], and no report exists that investigated alterations in aging biomarkers, attributed to chemotherapy, show an impact on clinical outcome. Therefore, we prospectively compared clinical and as well as potential biological aging markers in a cohort of older breast cancer patients given or not given chemotherapy after surgery. The patients were all >70 years of age, thus investigating a truly 'older' population. In those patients, geriatric assessment often reveals previously unknown age-related problems [2].

After 1 year of follow-up, we found that chemotherapy did not significantly influence established markers of clinical frailty. Our data only revealed a mild and transient decrease of global fitness status in older breast cancer patients undergoing chemotherapy: increased clinical frailty, as evidenced by a lower LOFS score, was noted in the CTG (but not CG) after 3 months of treatment, but the frailty status returned to baseline level after one year. Frailty status according to Balducci did not change during the time course of the study. Global QoL was also slightly decreased at 3 months in the CTG (but not the CG), but was also restored after 1 year. The temporary decrease in fitness and QoL is not unexpected, and can be explained by acute and subacute chemotherapy toxicity. However, our study has proven that overall, the TC regimen [41-43] (generally administered with primary G-CSF support) is well tolerated in older breast cancer patients. Apart from febrile neutropenia (13% grade III), we noticed few grade III, and even no grade IV side effects.



* Designates statistical significant (p≤ 0.05) differences at T1 or T2, compared to T0, within the Chemo group (green asterisk) or the Control group (blue asterisk)

A. Mean Leukocyte Telomere Length (+95% CI) by Time and Study Arm
B. Mean IL-6 (+95% CI) by Time and Study Arm (based on log transformed data, UNIT pg/mL)
C. Mean IL-10 (+95% CI) by Time and Study Arm (based on square root transformed data, UNIT pg/mL)
D. Mean TNF-α (+95% CI) by Time and Study Arm (based on log transformed data, UNIT pg/mL)
E. Mean IGF-1 (+95% CI) by Time and Study Arm (based on log transformed data, UNIT ng/mL)
F. Mean MCP-1 by Time and Study Arm (based on log transformed data, UNIT pg/mL)
G. Mean RANTES by Time and Study Arm (based on log transformed data, UNIT pg/mL)

Figure 1: Evolution over time of aging biomarker results in the Chemo and Control Groups. * Designates statistical significant (p≤ 0.05) differences at T1 or T2, compared to T0, within the Chemo group (green asterisk) or the Control group (blue asterisk) **A.** Mean Leukocyte Telomere Length (+95% CI) by Time and Study Arm **B.** Mean IL-6 (+95% CI) by Time and Study Arm (based on log transformed data, UNIT pg/mL) **C.** Mean IL-10 (+95% CI) by Time and Study Arm (based on square root transformed data, UNIT pg/mL) **D.** Mean TNF-α (+95% CI) by Time and Study Arm (based on log transformed data, UNIT pg/mL) **E.** Mean IGF-1 (+95% CI) by Time and Study Arm (based on log transformed data, UNIT ng/mL) **F.** Mean MCP-1 by Time and Study Arm (based on log transformed data, UNIT pg/mL) **G.** Mean RANTES by Time and Study Arm (based on log transformed data, UNIT pg/mL).

Table 3: Geriatric assessment results at baseline (T0), 3 months (T1), and 1 year (T2), and their differential evolution over time in Chemo and Control Groups

	Chemo Group (n=57)			Evolution Over Time Chemo Group		Control Group (n=52)			Evolution Over Time Control Group		Differential Evolution (Time Interaction)
	T0	T1	T2	T0→T1	T0→T2	T0	T1	T2	T0→T1	T0→T2	
Frailty(Balducci) N Fit n (%) Vulnerable n (%) Frail n (%)	56 12 (21) 21 (35) 23 (41)	53 8 (15) 23 (43) 22 (42)	48 10 (21) 15 (31) 23 (48)	p=0.87	p=0.41	52 10 (19) 17 (33) 25 (48)	48 9 (19) 13 (27) 26 (54)	46 7 (15) 13 (28) 26 (57)	p=0.34	p=0.77	*p*=0.63α
LOFS N Mean +/- SD	56 7.5 +/- 2	53 6.7 +/- 2	48 7.4 +/- 2	**p<0.01**	*p*=0.60	51 6.8 +/- 2	48 7.0 +/- 2	46 6.8 +/- 2	p=0.48	p=0.45	**p<0.01**
ADL N Mean +/- SD 6§ n (%) 0-5 n (%)	56 5.5 +/- 1 33 (59) 23 (41)	56 5.4 +/- 1 35 (62) 21 (37)	51 5.5 +/- 1 30 (59) 21 (41)	p=0.88 *p*=0.88	p=0.96 *p*=0.99	52 5.1 +/- 1 29 (56) 23 (44)	48 5.0 +/- 1 23 (48) 25 (52)	46 5.0 +/- 1 21 (46) 25 (54)	p=0.94 *p*=0.85	p=0.57 *p*=0.47	p=0.77 *p*=0.76
IADL N Mean +/- SD 8§ n (%) 0-7 n (%)	57 6.6 +/- 2 24 (42) 33 (58)	56 6.0 +/- 2 16 (29) 40 (71)	51 6.8 +/- 2 26 (51) 25 (49)	**p<0.01** *p*=0.01	p=0.39 *p*=0.39	52 5.8 +/- 2 17 (33) 35 (67)	48 5.8 +/- 2 17 (35) 31 (65)	46 5.7 +/- 3 17 (37) 29 (63)	p=0.71 *p*=0.75	p=0.57 *p*=0.60	**p<0.01** *p*=0.01
Previous falls N No n (%) Yes n (%)	57 44 (77) 13 (23)	N/A	50 34 (68) 16 (32)	N/A	p=0.28 *p*=0.25	52 28 (54) 24 (46)	N/A	46 29 (63) 17 (37)	N/A	p=0.33 *p*=0.37	p=0.15β *p*=0.15 β
MMSE N Mean +/- SD	57 27.9 +/- 2	56 27.6 +/- 3	51 27.9 +/- 3	p=0.22 *p*=0.19	p=0.94 *p*=0.96	52 27.9 +/- 2	48 27.8 +/- 3	46 27.9 +/- 4	p=0.34 *p*=0.31	p=0.40 *p*=0.41	p=0.77 *p*=0.78
GDS-15 N Mean +/- SD 0-4§ n (%) 5-15 n (%)	55 2.9 +/- 2 46 (84) 9 (16)	56 3.1 +/- 2 43 (77) 13 (23)	50 3.1 +/- 3 38 (76) 12 (24)	p=0.31 *p*=0.23	p=0.50 *p*=0.51	46 2.8 +/- 2 35 (76) 11 (24)	47 3.5 +/- 3 32 (68) 15 (32)	45 3.1 +/- 3 35 (78) 10 (22)	p=0.23 *p*=0.25	p=0.40 *p*=0.40	p=0.98 *p*=0.98
MNA-SF N Mean +/- SD ≥ 12§ n (%) < 11 n (%)	57 11.1 +/- 2 25 (44) 32 (56)	55 9.9 +/- 2 16 (29) 39 (71)	50 11.5 +/- 2 27 (54) 23 (46)	**p<0.01** *p*<0.01	p=0.19 *p*=0.13	51 11.0 +/- 2 23 (45) 28 (55)	48 11.3 +/- 2 29 (60) 19 (40)	46 11.4 +/- 2 23 (50) 23 (50)	p=0.24 *p*=0.32	p=0.23 *p*=0.21	**p<0.01** *p*<0.01
CCI N Mean +/- SD 0§ n (%) 1 n (%) >=2 n (%)	54 0.6 +/- 1 32 (59) 12 (22) 10 (19)	53 0.7 +/- 1 32 (60) 10 (19) 11 (21)	49 0.7 +/- 1 28 (57) 12 (25) 9 (18)	p=0.42 *p*=0.48	p=0.16 *p*=0.78	52 1.1 +/- 2 27 (52) 12 (23) 13 (25)	50 1.1 +/- 2 27 (54) 10 (20) 13 (26)	46 1.2 +/- 2 22 (48) 11 (24) 13 (28)	p=0.86 *p*=0.97	p=0.35 *p*=0.91	p=0.630 *p*=0.86 θ
G8 N Mean +/- SD >14 n (%) ≤ 14 n (%)	56 14.2 +/- 2 28 (50) 28 (50)	NA	NA	N/A	N/A	52 13.7 +/- 2 22 (42) 30 (58)	NA	NA	N/A	N/A	N/A
Global QoL N Mean +/- SD	57 64.2 +/- 17	56 58.5 +/- 20	50 69.5 +/- 22	p=0.06 *p*=0.05	p=0.11 *p*=0.08	52 63.8 +/- 17	48 64.6 +/- 20	46 63.6 +/- 23	p=0.83 *p*=0.83	p=0.73 *p*=0.96	**p=0.02** *p*=0.03

Abbreviations. SD: Standard Deviation; CI: Confidence Interval; ADL: Activities of Daily Living; IADL: Instrumental Activities of Daily Living; MMSE: Mini Mental State Examination; GDS: Geriatric Depression Scale; MNA-SF: Mini Nutritional Assessment-Short Form; MNA: Mini Nutritional Assessment

§: Maximum score, no abnormalities

α: calculation based on probability of being fit or vulnerable by time and study arm

β: calculation based on probability of falling by time and study arm

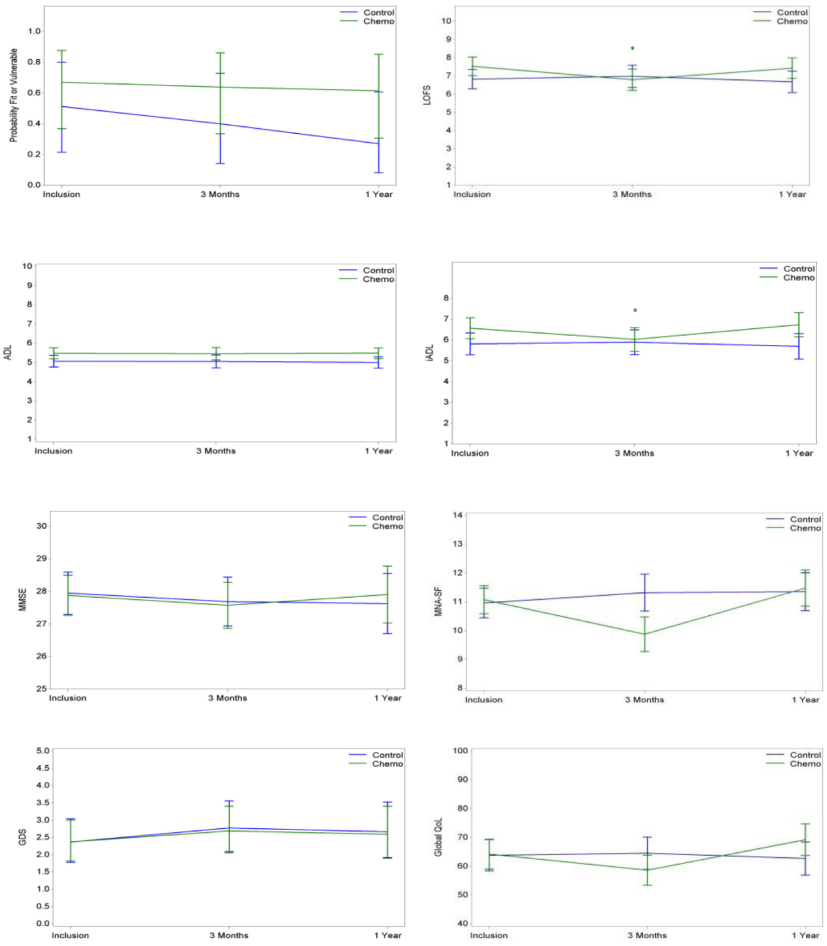
θ: calculation based on probability of having the lowest score at CCI, by time and study arm

p-values in italic font show results corrected for patient frailty level at baseline

Table 4: Correlation of baseline aging biomarkers with chronological age and clinical aging (according to LOFS).

	Chronological age (years)			Clinical aging (LOFS)		
	Spearman correlation	*p*-value	*N*	Spearman correlation	*p*-value	*N*
Telomere length	-0.11	0.32	86	-0.27	0.01	85
IL-6	0.32	<0.01	108	-0.21	0.03	106
IL-10	-0.03	0.78	101	-0.05	0.62	99
IGF-1	-0.01	0.33	106	-0.03	0.75	104
TNF-α	0.34	<0.01	108	-0.18	0.06	106
MCP-1/CCL-2	0.18	0.07	107	-0.14	0.16	105
RANTES/CCL-5	-0.01	0.88	107	0.16	0.10	105

Abbreviations. LOFS : Leuven Oncogeriatric Frailty Score; IL-6 : Interleukin-6; IL-10 : Interleukin-10; IGF-1 : Insulin Like Growth Factor-1; TNF-α : Tumor Necrosis Factor-α; MCP-1/CCL2 : Monocyte chemotactic protein-1/Chemokine (C-C motif) ligand 2; RANTES/CCL5 : Regulated Upon Activation, Normal T cell Expressed and presumably Secreted/ Chemokine (C-C motif) Ligand 5



* Designates statistical significant (p≤ 0.05) differences at T1 or T2, compared to T0, within the Chemo group (green asterisk) or the Control group (blue asterisk)

A. Predicted probability (+95% CI) of being 'fit or vulnerable' by Time and Study arm, by Balducci
B. Mean Leuven Oncogeriatric Frailty Score (LOFS) (+95% CI) by Time and Study arm
C. Mean score for Activities of Daily Living (ADL) (+95% CI) by Time and Study arm
D. Mean score for instrumental Activities of Daily Living (iADL) (+95% CI) by Time and Study arm
E. Mean score for Mini Mental State Evaluation (MMSE) (+95% CI) by Time and Study arm
F. Mean score for Mini Nutritional Assessment Short Form (MNA-SF) (+ 95% CI) by Time and Study arm
G. Mean score for Geriatric Depression Scale (GDS) (+95% CI) by Time and Study arm
H. Mean Global Quality of Life (+95% CI) by Time and Study arm

Figure 2: Evolution over time of geriatric assessment parameters in the Chemo and Control Groups. * Designates statistical significant (p≤ 0.05) differences at T1 or T2, compared to T0, within the Chemo group (green asterisk) or the Control group (blue asterisk) **A.** Predicted probability (+95% CI) of being 'fit or vulnerable' by Time and Study arm, by Balducci **B.** Mean Leuven Oncogeriatric Frailty Score (LOFS) (+95% CI) by Time and Study arm **C.** Mean score for Activities of Daily Living (ADL) (+95% CI) by Time and Study arm **D.** Mean score for instrumental Activities of Daily Living (iADL) (+95% CI) by Time and Study arm **E.** Mean score for Mini Mental State Evaluation (MMSE) (+95% CI) by Time and Study arm **F.** Mean score for Mini Nutritional Assessment Short Form (MNA-SF) (+ 95% CI) by Time and Study arm **G.** Mean score for Geriatric Depression Scale (GDS) (+95% CI) by Time and Study arm **H.** Mean Global Quality of Life (+95% CI) by Time and Study arm

Table 5: Cumulative toxicity

Cumulative adverse event		Chemotherapy Group /Control group Grade in %			
		1	2	3	4
Febrile neutropenia	T0 → T1	0 / 0	0 / 0	13 / 0	0 / 0
	T1 → T2	0 / 0	0 / 0	0 / 0	0 / 0
Anemia	T0 → T1	63 / 18	11 / 8	2 / 0	0 / 0
	T1 → T2	18 / 25	2 / 4	0 / 0	0 / 0
Diarrhea	T0 → T1	26 / 2	4 / 0	2 / 0	0 / 0
	T1 → T2	2 / 0	2 / 0	0 / 0	0 / 0
Nausea/vomiting	T0 → T1	41 / 2	7 / 0	0 / 0	0 / 0
	T1 → T2	0 / 4	0 / 0	0 / 0	0 / 0
Anorexia	T0 → T1	46 / 6	9 / 0	2 / 0	0 / 0
	T1 → T2	6 / 2	6 / 4	0 / 0	0 / 0
Fatigue	T0 → T1	44 / 26	22 / 0	2 / 2	0 / 0
	T1 → T2	30 / 19	2 / 4	2 / 0	0 / 0
Pain	T0 → T1	30 / 28	9 / 2	0 / 0	0 / 0
	T1 → T2	40 / 53	4 / 2	0 / 0	0 / 0
Mucositis	T0 → T1	24 / 0	2 / 0	2 / 0	0 / 0
	T1 → T2	1 / 0	0 / 0	0 / 0	0 / 0
Sensory neuropathy	T0 → T1	15 / 4	0 / 0	0 / 0	0 / 0
	T1 → T2	8 / 0	0 / 0	0 / 2	0 / 0
Rash	T0 → T1	17 / 0	0 / 0	0 / 0	0 / 0
	T1 → T2	2 / 0	0 / 0	0 / 0	0 / 0

The absence of a pronounced aging/frailty-inducing effect of chemotherapy was further corroborated by measurements of some of the principal well-established aging biomarkers, such as LTL and IL-6. LTL was comparable in both groups at baseline and progressively decreased over the 1-year time course of the study with no significant difference between the two groups. This is in line with the well-known age-related process of progressive telomere attrition [6, 7, 44] but does not support the hypothesis that the aging process is accelerated by chemotherapy. Similar to LTL, the plasma marker IL-6 [13] did not reveal chemotherapy-induced aging progression when considering the evolution over the 1-year study period. On the other hand, several of the additional plasma biomarkers that have previously been associated with aging [13, 15, 21, 22, 25, 27, 45-47], did suggest a slight aging-promoting effect of chemotherapy: decreases in IL-10 and IGF-1 and increases in TNFα and MCP-1 from baseline to 1 year were significantly more pronounced in the CTG compared to the CG, suggesting accelerated biological aging. However, we tend to consider the clinical impact of alterations in only a few biomarkers that contribute to the so-called 'inflammaging' phenomenon rather minimal, especially as more robust aging biomarkers do not appear to show the same trend.

Although geriatric assessment parameters and the patient's perception of QoL did not change significantly at 1 year, one might argue that clinical changes may not immediately become visible, but might remain subclinical for a longer period of time. On the other hand, it was shown by Benitez-Beluga et al. [37] that biological changes induced by chemotherapy can recover to normal after a sufficiently long period of follow-up. Therefore, the transient changes observed shortly after treatment in our study, seem not very likely to have any clinical significance on the long term.

Of all evaluated biomarkers, IL-6 showed the strongest correlation with chronological age and LOFS, confirming its robustness as an aging biomarker as previously described [13]. Associations of the other aging biomarkers were less prominent and mostly not significant. It should be noted, though, that the cohort examined in this study only comprised elderly people within narrow age range (70 – 90 years). Hence, the lack of association with chronological age in this study does not necessarily imply that these markers are not age-related at all.

From the clinical perspective, aging biomarkers that would be predictive for chemotherapy-associated adverse events (toxicity, unplanned readmissions), would be highly relevant. However, we found that none of the biomarkers tested was associated with grade II-III-IV toxicity or unplanned readmissions, and neither were the

clinical frailty scores (Balducci/LOFS)

Due to the non-randomized design of the study, we cannot exclude some selection bias. The CG was in fact slightly less fit than the CTG at the start of the study, as apparent from LOFS and biomarkers at baseline and by long-term frequency of hospitalization events (between T1 and T2). This was not unexpected, since not only patients at low risk for cancer recurrence, but also patients too frail for chemotherapy, were included as controls. However, this does not influence our conclusions, as we do not compare absolute values of test results at a specific time point, but rather consider differences in evolution over time of clinical and biological aging markers between the groups.

Taken together, we conclude that chemotherapy, after 1 year, does not significantly influence clinical aging parameters, nor does it induce an altered evolution in the most robust aging biomarkers recognized to date (i.e. LTL and IL-6). Nevertheless, other aging biomarkers (MCP-1, TNF-α, IL-10 and IGF-1) evaluated in this study indicated a (mild) potential aging promoting effect of chemotherapy. We found, however, no evidence that changes in these circulating molecules, as a consequence of chemotherapy, do result in clinically relevant changes in frailty, in morbidity, or in higher (all-cause) mortality.

Our study is the first to report a prospective comparison of exclusively older breast cancer patients receiving or not receiving post-operative chemotherapy by measuring several different clinical (GA) and biological aging markers. The results demonstrate that although some biological markers do change during and after chemotherapy, there is no convincing evidence of a clinically relevant acceleration of the aging process. This is an important finding because it emphasizes that chemotherapy should not be denied to older breast cancer patients solely because of their advanced age.

PATIENTS AND METHODS

Patient population and clinical assessment

This prospective, multicentre, non-interventional study accrued patients in 2 academic and 3 regional hospitals in Belgium from 2009 until 2012 (www.clinicaltrials.gov/ NCT00849758). Eligible patients for the chemotherapy group (CTG) were female, ≥70 years old with early invasive breast cancer for whom adjuvant chemotherapy was planned according to established risk factors and international guidelines [48]. The scheduled therapy consisted of docetaxel at a dose of 75 mg/m^2 and cyclophosphamide at 600 mg/m^2 every 3 weeks for a total of 4 cycles (TC scheme)[41-43]. Primary prophylaxis with G-CSF (granulocyte-colony stimulating factor) was administered as per standard practice guidelines. In parallel, we enrolled a control group (CG) consisting of early breast cancer patients ≥70 years old for whom chemotherapy was not indicated (or indicated, but judged not to be feasible), and who were administered an aromatase inhibitor as sole adjuvant systemic therapy. Patients either or not received adjuvant radiotherapy according to institution policy. In the chemotherapy group, patients with hormone sensitive tumors also received an endocrine therapy after completion of chemotherapy. Trastuzumab was associated to the adjuvant chemotherapy if the tumor was HER2 positive.

The study was approved by the Ethical Committee of the participating hospitals and written informed consent was obtained from all patients.

Patients were enrolled after surgery. They underwent blood sampling, geriatric assessment (GA) and Quality of Life (QoL) evaluation at three time points. The first time point was between 3 and 6 weeks after surgery, and always before the first chemotherapy administration. The second time point was approximately 3 months after inclusion (day of last chemotherapy), and the last time point was around 1 year after inclusion.

We performed a G8 [49]_ENREF_44 screening test[49] at baseline, and a GA, at each time point. Social data (age, living situation, marital status and educational level) were assessed. Functional status was measured by Katz's Activities of Daily Living (ADL) and by Lawton's instrumental Activities of Daily Living (iADL) scales. A fall history (number of falls during the previous 12 months and presence of fall-related injury) was recorded. We determined cognitive status with the Mini Mental State Examination (MMSE) and mood with the 15-item Geriatric Depression Scale (GDS-15). The nutritional status was assessed using the Mini Nutritional Assessment-Short Form (MNA-SF). Polypathology and severity of medical problems were measured with the Charlson Comorbidity Index (CCI). Geriatric scales have been described in detail by Kenis C. et al [50]. GA results were categorized into "fit", "vulnerable" and "frail" groups according to Balducci [51, 52]. However, as this categorisation has limitations (e.g. age above 85 is always considered "frail"), we developed a new scoring system, the *Leuven Oncogeriatric Frailty Score* (LOFS), to summarize GA results in a more refined linear score ranging from 10 (very fit) to 0 (very frail). Details are described in appendix 1, and in one of our previous publications [4].

Classical oncological parameters such as Eastern Cooperative Oncology Group - Performance Status (ECOG-PS), tumor characteristics (i.e. tumor subtype according St-Gallen criteria [53] and TNM) and treatment details were recorded. Adverse Events according to CTCAE v4.0 and unplanned readmissions were recorded. An unplanned readmission was identified as a subsequent or repeat hospitalization, which could not have been foreseen at the time of baseline time point [54].

Polypharmacy was assessed by the number of different registered drugs (www.bcfi.be) the patient had been taking during the week preceding inclusion. QoL was assessed with the EORTC QLQ-C30 questionnaire, from which the last two questions (question 29 and 30) were further used to determine 'global QoL'.

Blood sampling and measurement of aging biomarkers

At each time point, blood was sampled in 4-mL EDTA K2E tubes for plasma isolation and leukocyte DNA extraction.

Mean leukocyte telomere length (LTL) was measured on leukocyte DNA by qPCR [44] and plasma levels of IL-6, IL-10, IGF-1, TNF-α, MCP-1, and RANTES were assessed by ELISA. Detailed procedures are described in appendix 2.

Statistics and Endpoints

Statistics and endpoints are described in appendix 3.

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CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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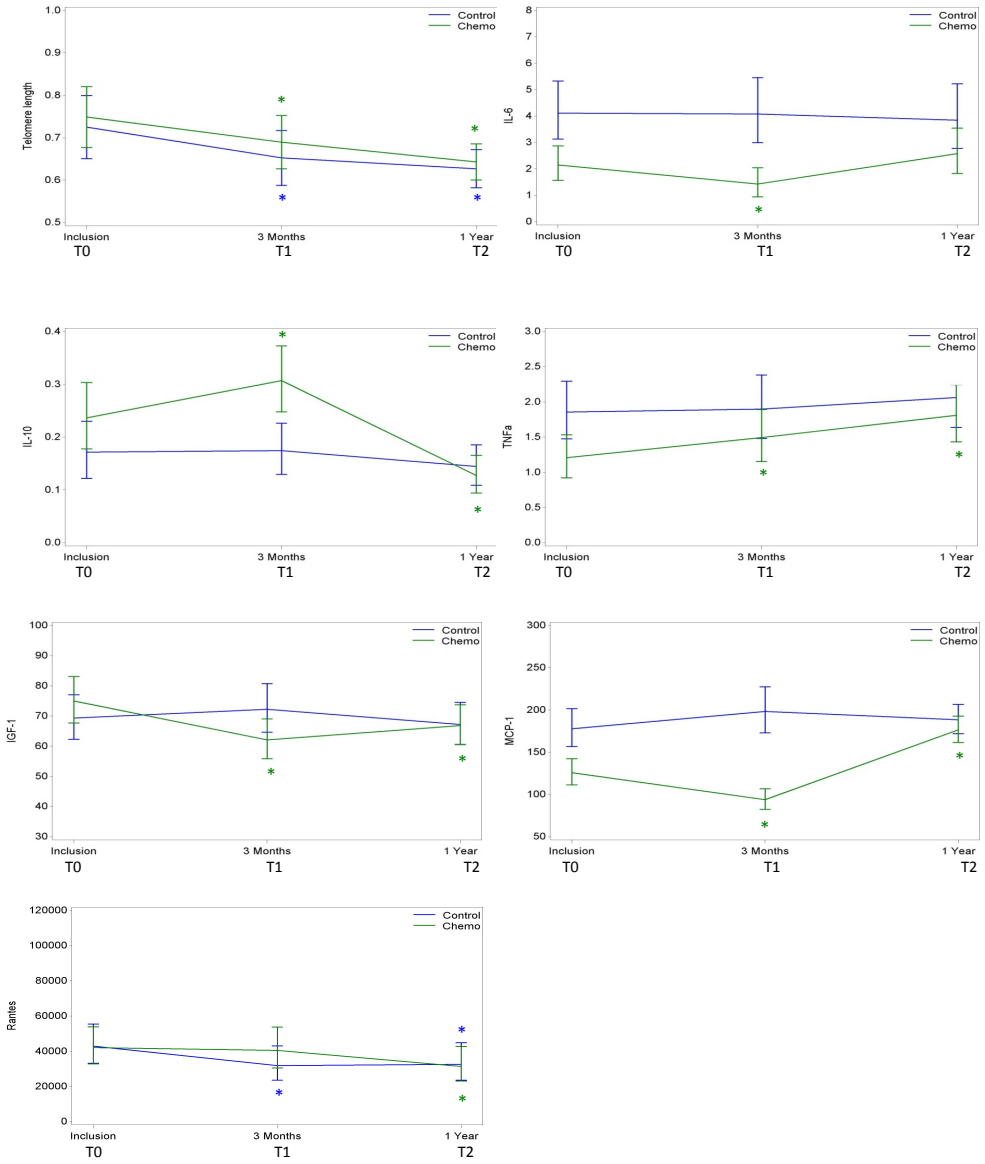
Figure 1.
Evolution over time of aging biomarker results in the Chemo and Control Groups.



* Designates statistical significant (p≤ 0.05) differences at T1 or T2, compared to T0, within the Chemo group (green asterisk) or the Control group (blue asterisk)

A. Mean Leukocyte Telomere Length (+95% CI) by Time and Study Arm
B. Mean IL-6 (+95% CI) by Time and Study Arm (based on log transformed data, UNIT pg/mL)
C. Mean IL-10 (+95% CI) by Time and Study Arm (based on square root transformed data, UNIT pg/mL)
D. Mean TNF-α (+95% CI) by Time and Study Arm (based on log transformed data, UNIT pg/mL)
E. Mean IGF-1 (+95% CI) by Time and Study Arm (based on log transformed data, UNIT ng/mL)
F. Mean MCP-1 by Time and Study Arm (based on log transformed data, UNIT pg/mL)
G. Mean RANTES by Time and Study Arm (based on log transformed data, UNIT pg/mL)

Figure 1.
Evolution over time of aging biomarker results in the Chemo and Control Groups.

* Designates statistical significant ($p \leq 0.05$) differences at T1 or T2, compared to T0, within the Chemo group (green asterisk) or the Control group (blue asterisk)

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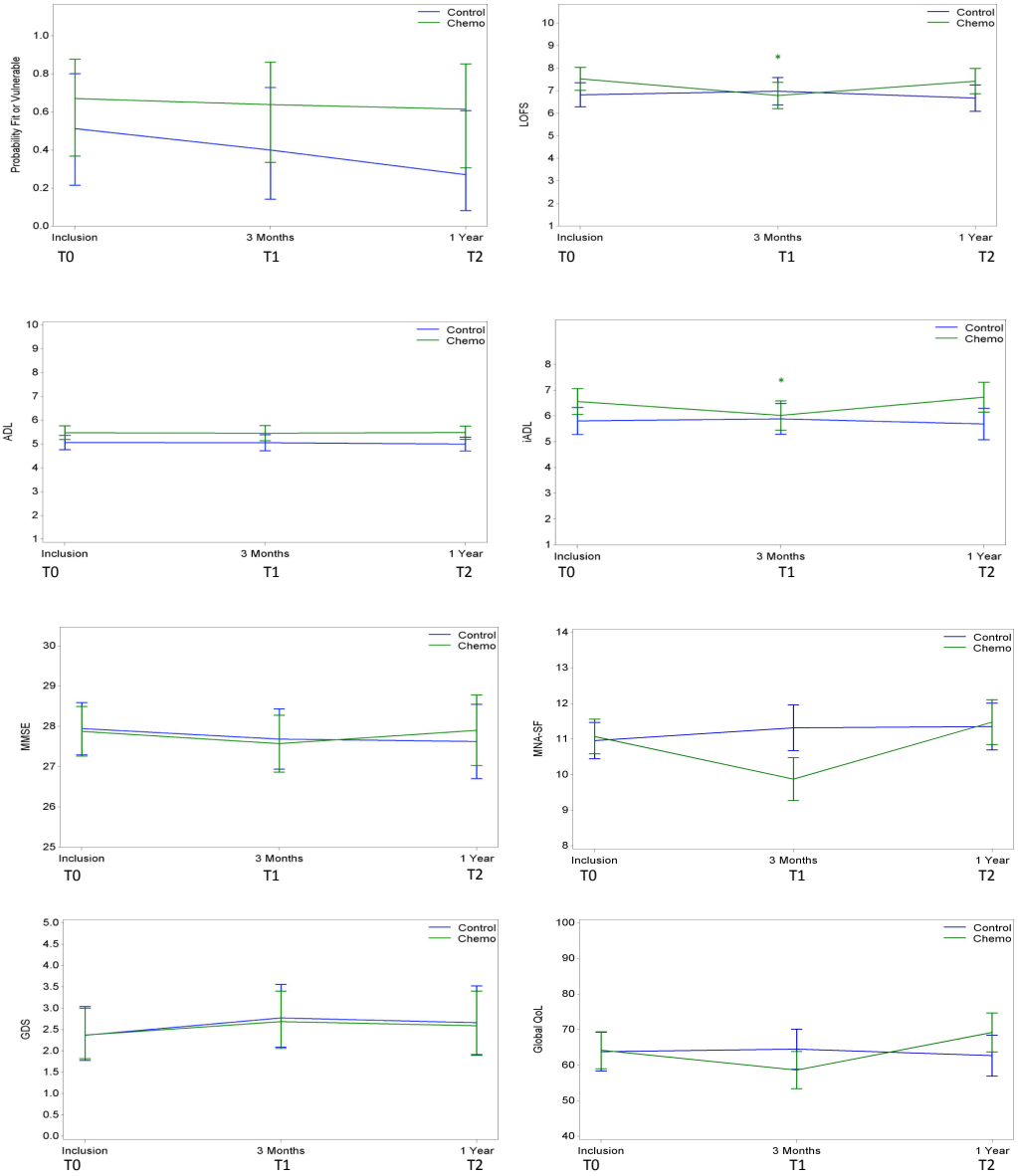
E. Mean IGF-1 (+95% CI) by Time and Study Arm (based on log transformed data, UNIT ng/mL)
F. Mean MCP-1 by Time and Study Arm (based on log transformed data, UNIT pg/mL)
G. Mean RANTES by Time and Study Arm (based on log transformed data, UNIT pg/mL)

Figure 2.
Evolution over time of geriatric assessment parameters in the Chemo and Control Groups.



* Designates statistical significant (p≤ 0.05) differences at T1 or T2, compared to T0, within the Chemo group (green asterisk) or the Control group (blue asterisk)

A. Predicted probability (+95% CI) of being 'fit or vulnerable' by Time and Study arm, by Balducci
B. Mean Leuven Oncogeriatric Frailty Score (LOFS) (+95% CI) by Time and Study arm
C. Mean score for Activities of Daily Living (ADL) (+95% CI) by Time and Study arm
D. Mean score for instrumental Activities of Daily Living (iADL) (+95% CI) by Time and Study arm
E. Mean score for Mini Mental State Evaluation (MMSE) (+95% CI) by Time and Study arm
F. Mean score for Mini Nutritional Assessment Short Form (MNA-SF) (+ 95% CI) by Time and Study arm
G. Mean score for Geriatric Depression Scale (GDS) (+95% CI) by Time and Study arm
H. Mean Global Quality of Life (+95% CI) by Time and Study arm

Figure 2.
Evolution over time of geriatric assessment parameters in the Chemo and Control Groups.

* Designates statistical significant ($p \leq 0.05$) differences at T1 or T2, compared to T0, within the Chemo group (green asterisk) or the Control group (blue asterisk)

A. Predicted probability (+95% CI) of being 'fit or vulnerable' by Time and Study arm, by Balducci
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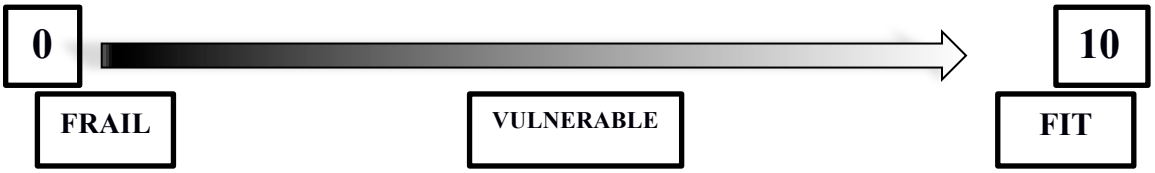
H. Mean Global Quality of Life (+95% CI) by Time and Study arm

Appendix 1
Leuven Oncogeriatric Frailty Score (LOFS)

	LOFS +2	LOFS +1	LOFS +0
ADL	6	5 - 4	≤ 3
iADL	8	7 - 4	≤ 3
MMSE	30 - 28	27 - 24	≤ 23
MNA-SF	14 - 12	11 - 8	≤ 7
CCI	0	1	≥ 2



LOFS is a semi-continuous frailty score which integrates results from Activities of Daily Living (ADL), instrumental activities of daily living (iADL), Mini Mental State Examination (MMSE), Mini Nutritional Assessment (MNA-SF) and Charlson Comorbidity Index (CCI). The scoring range for each separate test is separated in three groups, the lowest part (worst score range for each particular test) resulting in a LOFS +0 (no contribution to the final 10-points score), the middle part in +1 (contribution of 1 point), and the highest part in +2 (contribution of 2 points). Subscores from the 5 tests are added up to result in a total score on a scale that ranges from 0 (poorest score; extreme frailty) to 10 (best score, fit patient). Individual results from the LOFS should be interpreted as a gradation of severity in the spectrum of frailty between the two extremes.

Appendix 2 : Methods

Mean leukocyte telomere length (T/S ratio)

Mean leukocyte telomere length was measured on DNA of leukocytes extracted from the buffy coat remaining in the 4 ml EDTA tube after plasma removal. Every DNA sample was first tested for DNA fragmentation by electrophoresis on a 1% agarose gel. Fragmented DNA samples were excluded from further analysis. Telomere Length was assessed by qPCR. According to this method, the relative amount of telomeric DNA (T/S ratio) is calculated based on the Cp values obtained for telomeric DNA (T) and for the single-copy housekeeping gene 36B4 (S), measured in the same sample. All samples were assayed twice in independent qPCR runs, each time in triplicate wells. Each run included a dilution series (i.e. 80, 20, 5 and 1.25 ng) of standard DNA (Human Genomic DNA, Roche cat. no. 11691112001). The T/S ratio for an experimental sample is the amount (ng) of standard DNA that matches the experimental sample for copy number of the telomere template (T), divided by the amount (ng) of standard DNA that matches the experimental sample for copy number of the single-copy gene (S). Primer pairs used were 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3' and 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3' for telomeres and 5'-CAGCAAGTGGGAAGGTGTAATCC-3' and 5'-CCCATTCTATCATCAACGGGTACAA-3' for 36B4. The reaction mixture included 1x LightCycler 480 SYBR Green I Master, telomere primers at 0.6 µM each or 36B4 primers at 0.5 µM each, and 20 ng of template DNA in a total volume of 20 µL. Plates were run on a Roche LightCycler 480 platform, using the following thermal cycling program : activation for 10 min at 95°C; two initiation cycles of 15s at 95°C followed by 15s at 49°C; 35 amplification cycles of 15s at 95°C, 10s at 60°C and 15s at 72°C. Melting curves were then established in order to check amplicon purity.

Measurements of molecules in plasma

Plasmatic levels of cytokines, chemokines and IGF-1 were analyzed by ELISA method following manufacturers' instructions.

IL-6 , IL-10, TNF-alfa, CCL5/RANTES, CCL2/MCP-1, and IGF-1 levels were measured with Quantikine ELISA kit (R&D Systems)

Read-out was performed by dual spectrophotometric measurement: absorbance measured at 570 nm was subtracted from absorbance measured at 450 nm for IL-6, RANTES, MCP-1 and IGF-1. For TNF-alfa and IL-10, absorbance measured at 690 nm was substracted for absorbance measured at 490 nm. All samples were assayed in duplicate. On each microplate, a standard curve, obtained from dilution of a standard with known concentration, was included. From these standard curves concentrations of samples were calculated by a logistic curve-fitting algorithm.

Appendix 3 : Statistics and Endpoints

Statistics

For the primary endpoint, a linear model for longitudinal data was used with telomere length as response variable and time, study arm and their interaction as explanatory variables. An unstructured residual covariance matrix was modelled to account for clustering by repeated measures. For the secondary endpoints, linear models for longitudinal data were used for continuous responses, analogous to primary endpoint analysis. Analyses were performed on transformed responses where needed to improve symmetry of the distribution. Proportional odds models were used for ordinal responses, and logistic regression models for binary outcomes, both with random intercept to account for clustering by repeated measures. Spearman correlations were used for studying the association of aging markers with continuous variables. Kruskal-Wallis tests were performed to compare biomarker levels between more than 2 groups, and Mann-Whitney U tests were used for comparisons between two groups. All tests are two sided, and a 5% significance level is considered for all tests. All analyses have been performed using SAS software, version 9.3 of the SAS System for Windows. Copyright © 2002 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

Endpoints

The primary endpoint was to assess whether adjuvant chemotherapy for breast cancer induces accelerated telomere attrition. As secondary endpoints, we examined the impact of chemotherapy on plasma levels of IL-6, IL-10, IGF-1, TNF-α, MCP-1, and RANTES, on GA parameters, clinical frailty scores (LOFS and Balducci) and on QoL. Additionally, we investigated correlations of biological aging markers at inclusion with chronological age and clinical frailty.

Lastly, we investigated whether biological aging markers and clinical aging, at inclusion, were predictive for chemotherapy induced grade II-III-IV toxicity or unplanned readmissions.

1. Contribution of our work to the research field

The main purpose of this doctoral thesis was to broaden the scientific evidence on cancer in older people, both on the molecular level (focusing on the complex relationship between the aging process and the occurrence and evolution of cancer cells), as well as on the clinical level where we tried to clarify on important questions that are encountered by oncologists treating older cancer patients.

The aging breast cancer microenvironment

Oncology research over the last years has been focusing primarily on the cancer cells themselves. However, the influence of the stromal milieu is gaining more attention and has been shown to play an important role in the behavior of the cancer cells. Based upon this knowledge, and the known phenomenon of cellular senescence, research groups have been investigating age-related changes in fibroblasts, and their suspected influence on cancer cells.
Although the preclinical research on this topic has been showing very consistent findings, it is hampered by several important drawbacks that could have been introducing systematic bias in the results. First of all, most experiments start from fibroblasts in culture. Culture conditions are not in vivo conditions and could be influencing specifically some parameters of the senescence program (‘culture shock’ or ‘stress’ can induce senescence by itself). Secondly, by artificially inducing senescence in the cell cultures, these experiments have created an environment with an overload of senescent cells. This environment turned out in their results, to show cancer stimulatory effects. However, this model is probably overestimating the in vivo frequency of senescent cells. Especially as we do only have limited data available on how much senescent fibroblasts accumulate in the body during aging. Most clinical data also seem to suggest a less aggressive disease course in older breast cancer patients, which is difficult to reconcile with the suggested negative influences of an older microenvironment.

Therefore, we felt that there was an urgent need to validate some key pathophysiological concepts on microenvironmental senescence, in the in vivo situation. We decided to design a study where fibroblasts were taken from a breast tumor without applying any further culture conditions, and to study the gene expression differences between very old, and very young breast cancer patients. The laser capture microdissection needed to perform this separation was a time-consuming procedure,

which explains the small sample size in the study. However, by validating our results on publically available datasets retrieved from other laser capture microdissection projects, we were able to confirm several of our findings, that most importantly, matched the hypothesis suggested in literature. We found a higher expression of genes responsible for proliferation, differentiation and migration in the stroma of older breast cancers. We found a higher expression of genes responsible for extracellular matrix stability in the younger breast cancer stroma. Although we only studied the mivroenvironment, and are therefore not entitled to make assumptions on the influence of this environment on the cancer cells, we can say that these results strongly suggest easier migration and invasion of cancer cells into the older microenvironment compared to the younger one.

As a second step, we decided to use a candidate gene approach to investigate whether we could find evidence for the presence of a senescence associated secretory profile and for autophagy in the older breast cancer stroma. Both processes have been described as important pathophysiological changes related to senescence in the microenvironment and suggested to be the most important reason why an older microenvironment is cancer promoting. The fact that we could validate the presence of both processes in our breast cancer samples represents the first in-patient evidence that these phenomena take place in the microenvironment of spontaneous occurring breast cancers.

Taken together, this is the first time that evidence for the presence of a senescence associated secretory profile and for autophagy in an older cancer microenvironment has been found in human cancer tissue. This is an important step in the further development of this research field.
We must note that autophagy in the stroma is part of a two-way process: high-energy metabolites resulting from stromal autophagy fuel cancer cells, but cancer cells can also cause stromal autophagy by themselves, through oxidative stress by producing hydrogen peroxide. We can't dissociate primary autophagy in the stroma as a result of senescence, from secondary autophagy caused by cancer cell influences. However, the fact that we find a significant enrichment in autophagy genes in the genes upregulated in older stromal samples shows that at least part of it must be explained by the difference in age.

An unanswered question is why we do not find convincing evidence for senescence in itself (the gene enrichment result being unsignificant for these groups of genes) in the older stromal samples. It could be explained by the fact that we start from only a limited number of samples, and that we have used only a few, very typical senescence genes to construct the signature. The value of the enrichment score that was calculated for the process of senescence was negative, suggesting enrichment of the genes in the older samples. But it failed to reach significance. We must not forget

either, that aging is a multifactorial process, and that other organismal changes related to the aging process could compensate for the tumor promoting characteristics of the microenvironment.

Biological and clinical parameters of aging

To answer our second an third research question, we performed a retrospective (Chapter 2) and a prospective study (Chapter 3), investigating the value of several biological markers in reflecting biological age, and subsequently using this information to interpret the influence of chemotherapy treatment on the older organism.

The most important finding of the retrospective study was the fact that interleukin-6, an established frailty biomarker in geriatric medicine has been confirmed as an important frailty marker in cancer patients. This is not trivial, as cancer is known to induce inflammatory reactions in the body, which questioned the validity of inflammatory aging markers in oncogeriatrics. Our study demonstrates that in the case of breast cancer these doubts are incorrect. Of course, we can't exclude that the cytokine levels have been influenced by the presence of the neoplasm. Adding a third cohort of older women without breast cancer would have allowed dissecting this scientific question. However our investigational purpose was pragmatic and focused on studying the validity of potential aging biomarkers in oncogeriatric patients. For this reason, and also because of practical limitations, we did not include a cancer-free population. Moreover most breast cancers- especially when diagnosed in a non-metastatic state- don't induce extreme cytokine responses. Our results prove that the changes induced by frailty overrule the smaller changes induced by the cancer itself. Results could be very different if the same study was repeated in tumors demosntrating important systemic repercussion, like for example metastatic small cell lung cancers.

Furthermore, we demonstrated that other potential aging biomarkers, like leukocyte telomere length, MCP-1 and IGF-1 show correlations with calendar age, but in this study we did not show any relationship of these markers with frailty.

In order to investigate the correlation of the biomarkers with clinical frailty, we used several approaches. We first of all used the internationally known classification of Balducci. As this is a simplified tool that ignores several nuances contained within the geriatric assessment, we tried to develop an alternative, more subtle method to summarize the level of frailty that results from a full geriatric assessment. We termed our new score LOFS, or Leuven Oncogeriatric Frailty Score. This score takes into account five of the crucial domains that are evaluated in a geriatric assessment: functional status for elementary daily activities (ADL) and for instrumental daily activities (iADL), cognition (MMSE), nutritional status (MNA-SF), and comorbidities (CCI), whereas the Balducci

categories do not include nutritional status, and do only include cognition from the start of dementia on (at the time it becomes a 'geriatric syndrome').
An important missing piece in this research part is the lack of a gold standard. Ideally, we want to find a key marker that reflects biological age (better than does chronological age) and that is easy and quick to measure. This could be a single marker, or a compilation of biomarkers e.g. an 'aging-signature'. But we do not have a gold standard to compare our potential markers to. As a surrogate, we use frailty. As explained in the introduction, frailty has several definitions, and although it represents a syndrome on it's own, it is also highly influenced by comorbidities. The only way to know if any biomarker (or a combination) does add on, or replaces the geriatric assessment in determining the biological age, would be to test it against end points like mortality or long term functional outcome. However, in a cancer population, where the cancer in itself can cause morbidity or mortality, these endpoints are again, biased.

For this reason, we did not exclude the biomarkers that did not reflect frailty, from our analysis in the prospective study on chemotherapy and aging. This study was designed with as primary endpoint: does chemotherapy accelerate biological aging? From the previous, it is clear that biological age can be estimated with several biological and clinical parameters, but that no gold standard exists. At the time the study started, we chose leukocyte telomere length as primary parameter, as it was the aging marker that was best established in geriatric literature with less influence from inflammatory status.
As answer to our primary question, we found that the leukocyte telomere length decreases over the follow up time of 1 year, but at the same pace in breast cancer patients receiving, or not receiving chemotherapy. We thus did not confirm previous reports mentioning a significant decrease in telomere length in patients treated with chemotherapy. We believe this is explained by several methodological shortcomings in previous studies, that we have tried to overcome in our own study. In the study of Lee et al[124], telomere length was found to decrease in 5 patients receiving chemotherapy for Non-Hodgkin Lymphoma. However, not only was the number of patients very limited, these patients displayed a wide age range (19 years till 75 years old). They also received a variable number of chemotherapy cycles, and the chemotherapy scheme varied between patients. Moreover, the conclusion that decline in telomere length was due to therapy was made through comparison of the mean leukocyte telomere length in cancer patients after chemotherapy, with the mean leukocyte telomere length measured in an unrelated, healthy age-matched group at an arbitrary time-point. As telomere length is highly variable between patients, even of the same ages, comparison of absolute mean values between small patient groups of very different ages does not allow making sound conclusions. The next study on telomere length dynamics under chemotherapy,

was performed by Unryn et al[120]. They found faster telomere shortening in patients receiving chemoradiotherapy for head and neck cancer compared to historical controls. The investigated timespan was only 28 days and the controls were again suboptimal (control subjects were recruited by random digit dialing and who agreed to provide one blood sample and some personal information). A third study investigating telomere length evolution, found that telomere length in breast cancer patients receiving chemotherapy only transiently decreases, with recovery to normal age-expected values at 2 years after chemotherapy[125]. These age-expected values were calculated based on a healthy donor group. Telomere length before and after treatment, was obtained in a cross-sectional way, so the mean telomere length before treatment, was obtained from other patients than those included in the group that yielded the mean telomere length value during or after treatment. No comparison with the evolution in matched cancer patients treated without chemotherapy was made. The study of Sanoff et al[126] did not show increased telomere shortening in patients having received chemotherapy. But no control group was included in this study, comparisons on telomere dynamics were made using historical controls, and even using data obtained from murine experiments.

Taken together, none of the previously published studies on telomere evolution in chemotherapy treated patients included a prospectively collected control group of cancer patients. Most conclusions were made based upon comparison with historical controls, or healthy volunteers. Sometimes, the age ranges of the patients differed greatly, and some studies did not even compare the evolution of senescence markers within the same patients (at different time points), but did compare absolute values between different patients taken at a fixed time point in a cross sectional way.

In our study, we have built a design trying to avoid some important methodological pitfalls. We selected our breast cancer patients older than 70 years of age at diagnosis in order to focus on the older population. The study group consisted of patients receiving chemotherapy (all the same schedule by intention to treat, although ultimately, a few patients switched to another schedule or quit therapy due to allergic reactions). Most importantly, we selected a control group that also had early breast cancer and also had undergone the same surgical treatment. The only difference with the study patients was that the controls were not advised chemotherapy. Herein lies the strength of our study: none of the previous studies has managed to obtain samples from a control group consisting of comparable cancer patients. Some studies compared to historical controls, others selected a healthy age matched group as controls. Their results might have been biased by the

difference in morbidity between groups, and also by the difference in previous or ongoing treatments like surgery, radiotherapy, …
Ideally, our patient groups would have been randomized. Due to ethical considerations, it was however not possible to randomize patients to either or not chemotherapy. This resulted in a slightly less fit control group, as apparent from LOFS and biomarkers at baseline and by long-term frequency of hospitalization events. This was not unexpected, since not only patients at low risk for cancer recurrence, but also patients too frail for chemotherapy, were included as controls. We believe that this does not impact our conclusions as we did not compare absolute values of telomere length at a specific time point, but rather considered differences in evolution over time between the groups.

Concluding on telomere length, we can state that we did not find a significant increase in telomere shortening in the group of patients receiving chemotherapy.

We then looked at evolution of other biomarkers to see if their evolution could corroborate the conclusion on telomere length. IL-6, the marker that correlated best with frailty assessment in our retrospective study, confirmed our conclusion: there was no chemotherapy-induced difference measurable at 1 year of follow up.
Four of the other biomarkers however, did suggest a slight age-promoting effect: IL-10 and IGF-1 decreased and TNF-α and MCP-1 increased in the chemotherapy group (all at timepoint 1 year), while their values remained more or less stable in the control group.
The fifth biomarker, RANTES, showed a similar decrease in chemotherapy and control group.

As we commented on the retrospective study, we mention here again the potential value of including a cancer-free older control group as third party. It would have added to the scientific information on cytokine and chemokine differences induced by the presence of an oncological disease, compared to the differences induced by the prescribed cancer treatment. But given the results of our retrospective study we believe that the inflammatory changes related to breast cancer do not overrule the changes related to aging. In this specific prospective study setting, the tumor had been removed surgically before inclusion, and patients were recovering from surgery, waiting for radiotherapy in most cases. For making assumptions on the effect of chemotherapy on the aging process, we needed control patients that undergo comparable stressors to exclude as much bias as possible.

We can't provide an unambiguous rationale explaining the paradoxical findings on telomere length, RANTES and IL-6 on the one hand, and the other biomarkers on the other hand. We tend to consider the impact of alterations in only a few biomarkers that contribute to the so-called 'inflammaging' phenomenon rather minimal, especially as the more robust aging biomarkers (telomere length and IL-6) do not appear to show the same trend. All the more because we did not find any clinical signs of accelerated aging at 1 year follow up. Any changes in geriatric assessment and quality of life assessment that were noticed at 3 months follow up (and that can be explained by the short-term toxicity of chemotherapy), disappeared at 1 year. This highlights another strength of our study: next to the panel of biomarkers that was measured, we performed repeated geriatric assessments and quality of life questionnaires in all patients to measure the impact of chemotherapy on the frailty level and overall quality of life. As we do not have a gold standard measuring biological age, we believe that conclusion must not be based on biological markers alone, but must take into account clinical correlates. This clinical part was not included in any of the previously published studies.

After our prospective study started recruiting patients, the paper by Sanoff et al[126] was published. They did not only measure telomere length as mentioned previously in this discussion, but they also measured the expression of $p16^{INK4A}$ in peripheral blood T-lymphocytes. An increase in $p16^{INK4A}$ expression after 1 year was found in chemotherapy treated patients, compatible with acceleration of the aging process by chemotherapy. There was no control group included, but expected $p16^{INK4a}$ expression values for age were inferred from historical controls and in vitro data. To confirm their findings, the authors performed an independent analysis in a cross-sectional cohort of 176 breast cancer survivors enrolled after treatment, from which 34% received chemotherapy. This cross-sectional analysis showed a maintained increase in $p16^{INK4A}$ expression in chemotherapy treated patients, at a median 3.4 years after treatment. The methodological limitations of this study have already been highlighted. We can add to this that the cross-sectional validation could carry a selection bias: more frail patients that did not receive chemotherapy could already have died by the time they could have been included in the cross sectional validation, favoring the non-chemotherapy group in the $p16^{INK4A}$ measurements. Patients that relapsed from their breast cancer and received new chemotherapy for this relapse will also have been excluded, potentially introducing bias. A prospectively collected control group minimizes this kind of bias. Most importantly, we would have liked to see the clinical repercussion of the described biochemical changes, and the head-to-head comparison with a similar cancer patient group not receiving chemotherapy. On our own study samples, we evaluated the possibility of measuring the expression of $p16^{INK4A}$ in peripheral blood. As we did not plan upfront to measure $p16^{INK4A}$ expression we only decided to collect whole blood

RNA throught Paxgene® tubes, and not separate T-lymphocytes immediately after sample collection. After the study had already started, we decided to try to measure $p16^{INK4A}$ expression (on the total RNA), and extract mathematically how much of this expression could be attributed to the lymphocytes, by using the white blood cell formula. Unfortunately, our Paxgene tubes did not yield enough RNA in most patients, in order to perform this RT-qPCR, which required a high RNA input. That is why we could not investigate $p16^{INK4A}$ expression changes in our study.

Further follow up on the patients in our prospective study will provide clarity whether we can link any of the clinical or biological markers to differences in overall survival. Despite the useful information that will come from this follow up, we will also have to take into account the cancer-specific mortality, which might make it difficult to attribute differences in mortality entirely to biological aging. Adding a healthy age-matched population would in this regard have been interesting.

The main purpose of our study was trying to evaluate the effect of chemotherapy on biological aging, which would be compatible with chemotherapy toxicity on the longer term. We also performed measurements of biomarkers and geriatric assessment at 3 months, and collected toxicity data, in order to evaluate as a secondary endpoint, if one of the clinical or biological aging parameters would be predictive of short-term chemotherapy toxicity (grade II-III-IV) in older patients. As explained in the introduction, two predictive scoring systems have been developed that attempt to predict toxicity of chemotherapy in an older cancer population: the CRASH score[113] and the Hurria score[114]. Neither one of the scoring systems however, have managed to get implemented into routine clinical practice. Probably because oncologists are not familiar with the Chemotox value used in the CRASH score, and because of the complexity of the Hurria score. Moreover, the value of these scores for the individual patient is questionable, as they have been developed on a heterogeneous cancer population (different cancer types together).
Apart from febrile neutropenia (13% grade III), we noticed only few grade III, and even no grade IV side effects in our chemotherapy population. None of the biomarkers measured at the start of treatment, were predictive for toxicity or unplanned readmissions, and neither were the clinical frailty scores (Balducci/LOFS). To this secondary endpoint, we must conclude that biomarkers of aging can't be used as prediction for short-term toxicity in breast cancer patients treated with Docetaxel-Cyclophosphamide.

In final conclusion to this research project, we state that:

- Aging in the host translates into differences in stromal characteristics in breast cancers.

- IL-6 can be used as frailty biomarker in older breast cancer patients, and leukocyte telomere length, MCP-1 and IGF-1 reflect increasing age in this population.
- Based on the most robust biomarkers of aging (Leukocyte Telomere Length and IL-6) as well as on geriatric assessment results, there is no evidence that chemotherapy with Docetaxel-Cyclophosphamide would accelerate the rate of biological aging in older breast cancer patients
- Neither biomarkers of aging, nor geriatric assessment parameters, are predictive for short-term chemotherapy induced toxicity.

2. Future perspectives

This research project has opened up several new, intriguing questions.

The aging stroma

We have further corroborated the fact that aging in the host is responsible for changes in tumoral stroma, and preclinical evidence exists that these changes would be responsible for a different tumoral behavior. Our findings do only represent a small step in the entire staircase that must be climbed to understand the complex and intense link between cancer and aging. It would be interesting to match gene expression data from stroma, with gene expression data from the corresponding tumor cells. We have now shown that the stroma displays different gene expression characteristics, but by performing gene expression analysis on the corresponding tumor cells, we could investigate if this matches gene expression changes in the tumoral compartment.
Furthermore, investigating the epigenetics in stroma and tumor DNA could provide further clarification on the mechanism by which the gene expression differences are caused.
For this purposes, we have kept the tumor nests that were left after microdissection of the stroma. In a next research project, this could be used to extract RNA or DNA.

Biomarkers of aging and chemotherapy influence on the aging process

We have used a comprehensive panel of potential aging biomarkers, but several other biomarkers can be considered to evaluate the influence of chemotherapy on the aging process. In the past, we have published on MicroRNA's as easy-to-measure biomarkers of aging in breast cancer patients[129], and measurements of several of these MicroRNA's are currently being run on the samples of our prospective study. Signs of oxidative stress in the plasma and proteins reflecting telomere damage

(cathelicidin-related antimicrobial peptide or CRAMP, stathmin, Elongation Factor - 1α or EF-1α, and chitinase-3-like protein) are currently being evaluated on our retrospective study samples, and will be measured in the future on the prospectively collected samples further investigating chemotherapy-induced changes in aging biomarkers.
A specific topic concerns immune-aging: changes in circulating white blood cell subsets combined with CMV serostatus, has been shown specifically to reflect aging in the immune system. These parameters have been recently measured on whole blood samples that we collected together with our plasma, DNA and RNA samples during the prospective study, and data are now being analyzed.
For what concerns the measurement of $p16^{INK4A}$ expression, we will never be able to overcome the fact that we do not have specific T-lymphocyte RNA, but we are currently evaluating the possibility of amplifying the available RNA in order to perform expression analysis on the white blood cell RNA. These results can afterwards be corrected mathematically for the percentage of lymphocytes measured on the day of the blood drawing. In this way, we hope to be able to perform a (be it suboptimal) measurement of the evolution of $p16^{INK4A}$ expression during and after chemotherapy in our patients.

The ultimate goal of our research work is to improve cancer care for older patients and to provide more evidence-based guidelines for treatment decisions. Therefore we should gain more knowledge on the aging process, the interaction of the aging process with cancer, and the influence of cancer treatments on the aging process. We might ultimately find a combination of clinical and biological markers that quantifies the biological age of a person and helps us in making more justified treatment decisions.

G8

	Items	Mogelijke antwoorden	Score
A	Bent u afgelopen 3 maanden minder gaan eten als gevolg van verminderde eetlust, spijsverteringsproblemen, problemen bij kauwen en/of slikken?	**0** = belangrijk verlies van eetlust **1** = matig verlies van eetlust **2** = geen verlies van eetlust	……..
B	Gewichtsafname gedurende de 3 afgelopen maanden	**0** = gewichtsafname groter dan 3 kg. **1** = weet niet **2** = gewichtsafname tussen 1 en 3 kg. **3** = geen gewichtsafname	……..
C	Mobiliteit	**0** = aan bed of stoel gebonden **1** = in staat zelfstandig uit bed/stoel te komen, maar gaat niet naar buiten **2** = gaat zelfstandig naar buiten	……..
E	Neuropsychologische problemen	**0** = ernstig dement of depressief **1** = licht dement of depressief **2** = geen psychologische problemen	……..
F	BMI: (gewicht in kg) / (lengte in m2)	**0** = BMI <19 **1** = 19 ≤ BMI < 21 **2** = 21 ≤ BMI < 23 **3** = BMI ≥ 23	……..
H	Neemt de patiënt meer dan 3 geneesmiddelen?	**0** = ja **1** = neen	……..
P	Vindt de patiënt dat hij gezonder is, of minder gezond, dan de meeste mensen van zijn leeftijd?	**0,0** = minder gezond **0,5** = weet niet **1,0** = even gezond **2,0** = gezonder	……..
	Leeftijd	**0** = > 85 **1** = 80 - 85 **2** = < 80	……..
	Totaalscore (0-17)		……..

GRP

Omcirkel de juiste antwoordcategorie.

RISICO	JA	NEE
1. Aanwezigheid van een cognitieve stoornis	2	0
2. Alleenwonend of geen hulp mogelijk door inwonende partner/familie	1	0
3. Moeilijkheden bij stappen/transfers of gevallen in de afgelopen 6 maanden	1	0
4. Hij/Zij werd gehospitaliseerd in de afgelopen 3 maanden	1	0
5. De patient gebruikt ≥ 5 geneesmiddelen	1	0
Totaalscore (0-6)	.....	

Demografische gegevens

• Leeftijd • Geboortedatum	…….jaar |__|__| / |__|__| / |__|__|__|__|
• Geslacht	☐ Mannelijk ☐ Vrouwelijk
• Burgerlijke status	☐ Alleenstaand ☐ Getrouwd ☐ Gescheiden ☐ Weduwe / weduwnaar ☐ Andere:……..
• Woonsituatie	☐ Thuis: ☐ alleen ☐ met partner ☐ met familielid ☐ Service flat ☐ ROB / RVT / Woon- en zorgcentrum (WZC) ☐ Andere:……..
• Opleiding	❖ Naar school geweest tot de leeftijd van ……. jaar ❖ Specifieke informatie ☐ Basisschool ☐ Middelbare school ☐ Lager middelbaar (tot 15 jaar) Richting:…… ☐ Hoger middelbaar (tot 18 jaar) Richting:…… ☐ Hoger onderwijs Richting:…… ☐ Universitair onderwijs Richting:…… ☐ Ander:……

ADL

WASSEN	
☐ Krijgt geen hulp (stapt alleen in en uit bad of douche)	O
☐ Krijgt hulp bij baden voor het wassen van één lichaamsdeel (zoals rug of been)	O
☐ Krijgt hulp bij baden voor het wassen van meer dan één lichaamsdeel (of kan niet baden)	A
KLEDEN	
☐ Neemt kleding en kleedt zich alleen aan en uit zonder hulp	O
☐ Neemt kleding en kleedt zich alleen aan en uit zonder hulp, behalve voor het sluiten van schoenveters	O
☐ Krijgt hulp voor het nemen van de kledij of het zich kleden of blijft gedeeltelijk/volledig niet aangekleed	A
WC-GEBRUIK	
☐ Gaat alleen naar het toilet, reinigt zichzelf en trekt kledij terug aan zonder hulp (de persoon kan een hulpmiddel gebruiken zoals een wandelstok of rolstoel en een bedpan die hij 's morgens leegmaakt)	O
☐ Krijgt hulp om naar het toilet te gaan of om zich te reinigen of in het schikken van kledij of in het hanteren van de bedpan	A
☐ Gaat niet naar het toilet voor zijn uitscheiding	A
VERPLAATSEN	
☐ Gaat alleen in en uit bed, komt alleen in en uit de zetel (met eventueel gebruik van kruk / wandelstok)	O
☐ Krijgt hulp bij in en uit bed of zetel komen	A
☐ Komt niet uit bed	A
CONTINENTIE	
☐ Controleert urine en defecatiefunctie volledig zelf	O
☐ Occasionele accidentjes	A
☐ Supervisie helpt urine- of darmcontrole; blaassonde of incontinent	A

VOEDING □ Voedt zichzelf zonder hulp □ Eet alleen tenzij voor het snijden van het vlees of het smeren van de boterham □ Krijgt hulp bij voeding of wordt geheel of gedeeltelijk gevoed door maagsondes en/of infusen	 O O A
O = onafhankelijk / A = afhankelijk	

Aantal keer onafhankelijk (0-6)	……
Aantal keer afhankelijk (0-6)	……

IADL

	Score
GEBRUIK VAN DE TELEFOON	
☐ Bedient zich van de telefoon op eigen initiatief (zoekt de nummers op, kiest ze, enz.)	1
☐ Kiest slechts enkele welbekende nummers	1
☐ Neemt de telefoon op maar belt zelf niet op	1
☐ Gebruikt de telefoon helemaal niet	0
WINKELEN	
☐ Kan zelfstandig alle nodige boodschappen doen	1
☐ Is alleen voor sommige boodschappen zelfstandig	0
☐ Moet begeleid worden om boodschappen te doen	0
☐ Is volledig ombekwaam om boodschappen te doen	0
VOEDSELBEREIDING	
☐ Kan zelfstandig maaltijden plannen, bereiden en opdienen	1
☐ Kan geschikte maaltijden bereiden indien hij/zij van ingrediënten voorzien wordt	0
☐ Kan bereide maaltijden opwarmen en opdienen of kan maaltijden bereiden maar is niet in staat om het aangewezen dieet te volgen	0
☐ De maaltijden moeten voor hem/haar bereid en opgediend worden	0
HUISHOUDEN	
☐ Zorgt alleen voor het huishouden of doet het met occasionele hulp (bv. voor zwaar huishoudelijk werk)	1
☐ Voert lichte dagelijkse taken uit (zoals de vaat doen, het bed opmaken)	1
☐ Voert lichte dagelijkse taken uit maar op occasionele wijze	1
☐ Vergt hulp voor alle huishoudelijke taken	1
☐ Neemt helemaal niet deel aan de huishoudelijke taken	0

	Score
WASSEN	
□ Doet zijn/haar eigen was	**1**
□ Kan het kleine linnengoed wassen maar vergt hulp voor zwaarder linnengoed zoals lakens of handdoeken	**1**
□ De was moet door anderen gedaan worden	**0**
VERVOER	
□ Reist zelfstandig met het openbaar vervoer, de taxi of bestuurt eigen wagen	**1**
□ Gebruikt de taxi maar geen openbaar vervoer	**1**
□ Gebruikt het openbaar vervoer indien begeleid	**1**
□ De verplaatsingen zijn beperkt tot taxi of wagen met hulp van een derde	**0**
□ Verplaatst zich helemaal niet buitenshuis	**0**
GEBRUIK VAN GENEESMIDDELEN	
□ Neemt zelfstandig geneesmiddelen in op het gewenste uur en in de voorgeschreven dosis	**1**
□ Is in staat zelfstandig geneesmiddelen in te nemen, indien deze op voorhand klaargelegd worden	**0**
□ Is niet in staat om zelfstandig geneesmiddelen in te nemen	**0**
FINANCIEEL BEHEER	
□ Regelt zelfstandig de financiële zaken (budget, schrijft cheques uit, betaalt de huur en de facturen, gaat naar de bank).	**1**
□ Is in staat dagelijkse aankopen te doen maar heeft hulp nodig voor zijn/haar bankrekening of voor grote aankopen	**1**
□ Is onbekwaam om geldzaken te regelen	**0**
Totaalscore (0-8)	……

Valproblematiek

1. Algemeen

• Bent u gevallen in het afgelopen jaar? **INDIEN JA**: hoe vaak:……..	JA	NEE
• Heeft u letsels opgelopen ten gevolge van het vallen? **INDIEN JA**: welke letsels? □ ‘Mineure’ letsels □ ‘Majeure’ letsels	JA	NEE

2. Timed up and go test (TUG)

Resultaat TUG	……sec.	**Resultaat TUG cognitief**	……sec.

3. Valangst

- Hoe bang bent u dat u het komende jaar zou vallen en een letsel oplopen?
  - □ Zeer bang
  - □ Bang
  - □ Een beetje bang
  - □ Helemaal niet bang
- Zijn er zaken die u niet doet omdat u zou kunnen vallen?
  - □ Nee
  - □ Ja
- Zijn er zaken die u niet meer doet omdat u bang bent dat u zou kunnen vallen?
  - □ Nee
  - □ Ja

EORCT Qlq-C30

Gelieve alle vragen te beantwoorden door het getal te omcirkelen dat het meest van toepassing is. Er zijn geen 'juiste' of 'foute' antwoorden.

	Helemaal niet	**Een beetje**	**Nogal**	**Heel erg**
1. Heeft u moeite met het doen van inspannende activiteiten zoals het dragen van een zware boodschappentas of een koffer?	1	2	3	4
2. Heeft u moeite met het maken van een lange wandeling?	1	2	3	4
3. Heeft u moeite met het maken van een korte wandeling buitenshuis?	1	2	3	4
4. Moet u overdag in bed of in een stoel blijven?	1	2	3	4
5. Heeft u hulp nodig met eten, aankleden, u zelf wassen of naar het toilet gaan?	1	2	3	4

Gedurende de afgelopen week:

	Helemaal niet	Een beetje	Nogal	Heel erg
6. Was u beperkt bij het doen van uw werk of andere dagelijkse bezigheden?	1	2	3	4
7. Was u beperkt in het uitoefenen van uw hobby's of bij andere bezigheden die u in uw vrije tijd doet?	1	2	3	4
8. Was u kortademig?	1	2	3	4
9. Heeft u pijn gehad?	1	2	3	4
10. Had u behoefte te rusten?	1	2	3	4
11. Heeft u moeite met slapen gehad?	1	2	3	4
12. Heeft u zich slap gevoeld?	1	2	3	4
13. Heeft u gebrek aan eetlust gehad?	1	2	3	4
14. Heeft u zich misselijk gevoeld?	1	2	3	4
15. Heeft u overgegeven?	1	2	3	4
16. Had u last van constipatie?	1	2	3	4
17. Had u diarree?	1	2	3	4
18. Was u moe?	1	2	3	4
19. Heeft pijn u gehinderd in uw dagelijkse bezigheden?	1	2	3	4

Gedurende de afgelopen week:				
	Helemaal niet	**Een beetje**	**Nogal**	**Heel erg**
20. Heeft u moeite gehad met het concentreren op dingen, zoals een krant lezen of televisie kijken?	1	2	3	4
21. Voelde u zich gespannen?	1	2	3	4
22. Maakte u zich zorgen?	1	2	3	4
23. Voelde u zich prikkelbaar?	1	2	3	4
24. Voelde u zich neerslachtig?	1	2	3	4
25. Heeft u moeite gehad met het herinneren van dingen?	1	2	3	4
26. Heeft uw lichamelijke toestand of medische behandeling uw familieleven in de weg gestaan?	1	2	3	4
27. Heeft uw lichamelijke toestand of medische behandeling u belemmerd in uw sociale bezigheden?	1	2	3	4
28. Heeft uw lichamelijke toestand of medische behandeling financiële moeilijkheden met zich meegebracht?	1	2	3	4

Wilt u voor de volgende vragen het getal tussen 1 en 7 omcirkelen dat het meest op u van toepassing is							
	Erg slecht			**→**			**Uitstekend**
29. Hoe zou u uw algehele gezondheid gedurende de afgelopen week beoordelen?	1	2	3	4	5	6	7
30. Hoe zou u uw algehele "kwaliteit van het leven" gedurende de afgelopen week beoordelen?	1	2	3	4	5	6	7

MMSE

Oriëntatie:

	Maximum score	Score
In welk jaartal zijn we?	1	
In welk seizoen zijn we?	1	
In welke maand zijn we?	1	
Welke dag is het vandaag?	1	
De hoeveelste is het vandaag?	1	
In welk land leven we?	1	
In welke provincie zijn we nu?	1	
In welke stad zijn we nu?	1	
In welk ziekenhuis zijn we nu?	1	
Op welke verdieping zijn we nu?	1	

Inprentingsvermogen:

Wil je de voorwerpen die ik nu zal opnoemen, onthouden; en wil je ze herhalen als ik ze opgenoemd heb? BAL … VLAG … BOOM	3 aantal pogingen:	…….. ……..

Aandacht:

Wil je van het getal 100 zeven aftrekken, en trek van de uitkomst telkens weer 7 af, tot ik stop zeg. (93 86 79 72 65) … … … … … Wil je het volgende woord spellen? Het woord is DORST. Kun je het woord DORST nu van achteren naar voren spellen? (1 punt voor elke juiste letter op de juiste plaats)	*5* *5* hoogste score:	…….. ……..

Geheugen:

Wil je de namen van de 3 voorwerpen die we zopas hebben ingeoefend, herhalen? (bal, vlag, boom)	3	

Taal:

	Maximum score	Score
Wat is dit? (wijs een horloge aan) Wat is dit? (wijs een potlood aan)	1 1	
Wil je de volgende zin nazeggen: “Noch vis, noch vlees.”	1	
Wil je het volgende uitvoeren: “Neem een papier in je rechter-/linkerhand, plooi het dubbel, en geef het aan mij.”	3	
Ik ga je iets laten zien, lees wat er op het papier staat, en doe wat er gevraagd wordt. (sluit uw ogen)	1	
Wil je een korte zin opschrijven? (waaraan denk je nu?)	1	
Wil je deze figuren hier natekenen?	1	

Totaalscore (0-30)	……

Opmerkingen:

……

……

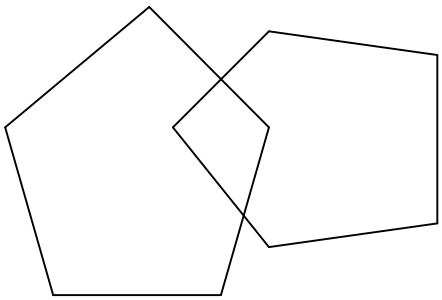
……

……

Sluit uw ogen

SCHRIJF EEN ZIN

TEKEN DIT NA



GDS

Kruis het antwoord aan dat het meest van toepassing is. Er zijn geen 'juiste' of 'foute' antwoorden.

	Ja	**Nee**
1. Bent u over het algemeen tevreden met uw leven?		
2. Heeft u veel van uw activiteiten en interesses laten vallen?		
3. Heeft u het gevoel dat uw leven leeg is?		
4. Verveelt u zich soms?		
5. Bent u meestal goedgezind?		
6. Bent u bang dat er u iets 'ergs' zal overkomen?		
7. Voelt u zich meestal gelukkig?		
8. Voelt u zich soms hopeloos?		
9. Blijft u soms liever thuis, dan uit te gaan en nieuwe dingen te doen?		
10. Heeft u het gevoel dat u meer moeilijkheden ondervindt met uw geheugen, dan de meeste andere mensen van uw leeftijd?		
11. Bent u blij nu te leven?		
12. Voelt u zich nogal waardeloos zoals u nu bent?		
13. Voelt u zich vol levensenergie?		
14. Heeft u het gevoel dat uw situatie hopeloos is?		
15. Denkt u dat de meeste mensen het beter hebben dan u?		
Totaalscore (0-15)	……	

MNA

Lengte	……..**cm**	**Gewicht**	……..**kg**

	Items	Mogelijke antwoorden	Score
A	Bent u afgelopen 3 maanden minder gaan eten als gevolg van verminderde eetlust, spijsverteringsproblemen, problemen bij kauwen en/of slikken?	**0** = belangrijk verlies van eetlust **1** = matig verlies van eetlust **2** = geen verlies van eetlust	……...
B	Gewichtsafname gedurende de 3 afgelopen maanden	**0** = gewichtsafname groter dan 3 kg. **1** = weet niet **2** = gewichtsafname tussen 1 en 3 kg. **3** = geen gewichtsafname	……...
C	Mobiliteit	**0** = aan bed of stoel gebonden **1** = in staat zelfstandig uit bed/stoel te komen, maar gaat niet naar buiten **2** = gaat zelfstandig naar buiten	……...
D	Hebt u gedurende de afgelopen 3 maanden last gehad van mentale spanning (stress) of acute ziekteverschijnselen?	**0** = ja **2** = neen	……...
E	Neuropsychologische problemen	**0** = ernstig dement of depressief **1** = licht dement of depressief **2** = geen psychologische problemen	……...
F	BMI: (gewicht in kg) / (lengte in m2)	**0** = BMI <19 **1** = 19 ≤ BMI < 21 **2** = 21 ≤ BMI < 23 **3** = BMI ≥ 23	……...
	Totaalscore (0-14)		……...

Charlson Comorbidity Index

Comorbiditeit	Aanwezig	Punten
Myocardinfarct		**1**
Decompensatie		**1**
Perifeer vaatlijden		**1**
Cerebrovasculaire aandoeningen (met uitzondering van hemiplegie)		**1**
Dementie		**1**
COPD		**1**
Autoimmuunziekte		**1**
Ulcus pepticum (slokdarm, maag, duodenum)		**1**
Matige leverfunctiestoornissen		**1**
Diabetes (zonder complicaties)		**1**
Diabetes met eindorgaan schade		**2**
Hemiplegie		**2**
Matige / ernstige nierfunctiestoornis		**2**
Tweede solide tumor (zonder metastasen)		**2**
Leukemie		**2**
Maligne lymfoom, Multiple myeloom		**2**
Matige / ernstige leverfunctiestoornis		**3**
Tweede solide tumor (met metastasen)		**6**
AIDS		**6**
Totaal punten (0-37)		……..

Polypharmacie

Gelieve alle geneesmiddelen te noteren die de patiënt genomen in de week voorafgaand aan de diagnosestelling.
Noteer telkens de eenheidsdosis die van toepassing is.

	Geneesmiddel	Eenheidsdosis vb. in mg
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		

Abstract of the Research

The main objective of this doctoral research was to expand the scientific knowledge on the interface between cancer and biological aging. Aging is a multifactorial process that is linked in a very complex way to cancer. Because this had never been done in tumors spontaneously occurring in human patients, we started by studying stromal characteristics of breast cancers arising in young respectively old patients. Based on the description of the SASP (senescence associated secretory profile) and AST (autophagy to senescence transition) in literature, two phenomena supposed to occur in senescent cells (e.g. fibroblasts), and the hypothesis that senescent cells accumulate in the body with aging, tumors arising in older patients could be expected to display a stromal compartment with different characteristics, which ultimately could lead to a different behavior of the tumor cells. By laser capturing the stromal compartments of breast cancers from young and old patients, and comparing the gene expression profiles, we confirmed for the first time in humans, the presence of both phenomena in the older breast cancer stromal samples. Moreover, we found that the older stromal compartment displays significant differences in gene expression compared to younger stroma, which concerned mainly genes responsible for proliferation, dedifferentiation and migration into the extracellular matrix.

In a second part of the thesis, we investigated biological aging in the rest of the organism, and the impact from cancer treatment (by chemotherapy) on this process. The main purpose of this research was to provide more evidence-based knowledge, allowing incorporation of the concept 'biological age' into therapy decisions for older patients. To do this, it was important to study the value of several biological markers in reflecting the biological age of a patient, as there is no current gold standard for this. In a retrospective study investigating several biological and clinical parameters of aging in young and old breast cancer patients IL-6 showed to be a robust frailty marker. Other markers like Leukocyte Telomere Length, IGF-1 and MCP-1 showed correlations with chronological age but not with frailty level. During this study we also developed the Leuven Oncogeriatric Frailty Score, a tool that summarizes the clinical frailty level of a patient in a more subtle way than do the currently used tools like Balducci classification.

Next, we performed a prospective study in early breast cancer patients either or not treated with adjuvant chemotherapy, and tested if the natural evolution of clinical and/or biological aging markers was influenced by chemotherapy. We did not find unexpected changes in the evolution of the most robust aging markers (Leukocyte Telomere Length and Interleukin-6) which means that we do not find convincing evidence that the chemotherapy we studied (Docetaxel-Cyclophosphamide) would accelerate biological aging in breast cancer patients. This is a reassuring finding for oncologists treating older patients. As a secondary endpoint, we checked if clinical or biological markers were correlated with short-term toxicity from chemotherapy, but neither of the aging parameters was useful in predicting grade II-III-IV toxicity, or unplanned hospital readmissions.

Dit doctoraatsonderzoek werd opgericht om meer wetenschappelijke inzichten te vergaren over de complexe verwevenheid van veroudering als biologisch process, en het optreden van kanker.
Eerst en vooral werd het micromilieu waarin borstkankercellen groeien onderzocht. Er werd nagegaan of dit micromilieu verschilt naarmate de leeftijd van de patient vordert. Er zijn verschillende wetenschappelijke gegevens voortkomend uit laboratoriumonderzoek, die dit suggereren. Anderzijds leert de klinische ervaring dat borstkanker bij oudere vrouwen meestal een trager en minder aggressief ziekteverloop kent.
We gebruikten de techniek van laser microdissectie om stukjes van dit micromilieu uit borstkankergezwellen van oudere en jongere patienten te snijden. Nadien onderzochten we verschillen in genexpressie op het bekomen materiaal. We stelden vast dat in het oudere micromilieu genen betrokken in celgroei, celmigratie en celdedifferentiatie meer tot expressie kwamen. Daarenboven werden enkele belangrijke concepten bevestigd: er bleken in het ouder micromilieu tekenen aanwezig van een ‘senescence associated secretory profile’, een fenomeen waarbij verouderde cellen allerlei proteines secreteren die tumorgroei kunnen bevorderen. Ook tekenen van autofagie-activatie werden aangetroffen in het oudere micromilieu. Ook dit kan tumorgroei kan stimuleren. Hoewel deze fenomenen reeds beschreven zijn in preklinisch onderzoek, hebben we dankzij dit onderzoek voor het eerst een bewijs geleverd van hun bestaan in patienten.
Verder onderzocht dit doctoraatsonderzoek ook het verouderingsproces in de rest van het lichaam, en meer specifiek het effect van een kankerbehandeling met chemotherapie hierop. Er bestaat momenteel geen goede test om de biologische leeftijd van een patient te meten.
We bestudeerden verschillende verouderings-merkers in een groep oudere en jongere borstkankerpatienten en vergeleken dit met hun resultaat op een geriatrische evaluatie, een test die de mate van hulpbehoevenheid op verschillende functionele vlakken probeert na te gaan. We vonden interleukine-6 als biologische merker die het best dit niveau van hulpbehoevendheid reflecteert. Enkele andere merkers bleken ook gerelateerd met de leeftijd van de patient.
In een laatste studie vergeleken we de evolutie van deze biologische merkers, alsook de evolutie van de geriatrisch evaluatie, in een groep borstkankerpatiënten die al dan niet behandeld werden met chemotherapie. We wilden nagaan of chemotherapie het verouderingsproces doet versnellen. We stelden geen veranderingen vast in evolutie van telomeerlengte of interleukine-6, noch in geriatrische evaluatie resultaten, bij patiënten die chemotherapie kregen. Andere biologische merkers suggereerden mogelijks een beperkt verouderingseffect van chemotherapie, doch gezien de meest krachtige verouderingsmerkers niet beinvloed blijken hierdoor, concluderen we dat er geen

overtuigende evidentie is dat chemotherapie doet verouderen.

Dr. Barbara Brouwers

Born 1982, Belgian nationality

- **Core Academic Education**

2015 - Current	Medical Oncologist at AZ Sint Jan Brugge, Belgium
2013 - 2015	5th and 6th year Specialization Medical Oncology, University Hospitals Leuven, Belgium
2009 - 2013	Research in the Lab of Experimental Oncology, KU Leuven – University Hospitals Leuven, Leuven, Belgium Granted twice by the Vlaamse Liga tegen Kanker (VLK) (Accounts for 3rd year specialization Internal Medicine and 4th year specialization Medical Oncology)
2008 - 2009	2nd year specialization General Internal Medicine, Imelda Ziekenhuis, Bonheiden
2007 - 2008	1st year Specialization General Internal Medicine, ZOL Genk
2007	Degree in Medicine, Katholieke Universiteit Leuven (KU Leuven), Belgium, Magna Cum Laude

- **Oral Presentations of the PhD research :**

- BSMO yearly meeting in 2012 and 2015
- Arnhem Oncologiedagen 2013
- ESMO young oncologist meeting 2015

- **Publications**

(* Equal contribution)

International Journals :

-**Barbara Brouwers**, Robert Paridaens, Jean-Pierre Lobelle, Wouter Hendrickx, Ann Smeets, Patrick Neven, Caroline Weltens, karen Deraedt, Isabelle Vanden Bempt, Hans Wildiers: Clinicopathological features of inflammatory versus non-inflammatory locally advanced non-metastatic breast cancer. Tumor Biol. 2008; 29(4):211-6

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-Bert Vandenberk*, **Barbara Brouwers***, Sigrid Hatse, Hans Wildiers: p16 INK4a: A central player in cellular senescence and a promising aging biomarker in elderly cancer patients. J Geriatric Oncology 2011; 2: 259-269. (* equal contribution)

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-Sigrid Hatse, Diether Lambrechts, Annemieke Verstuyf, Ann Smeets, **Barbara Brouwers**, Thijs Vandorpe, Olivier Brouckaert, Gilian Peuteman, Annouschka Laenen, Lieve Verlinden, Carsten Kriebitzsch, Anne-Sophie Dieudonné, Robert Paridaens, Patrick Neven, Marie-Rose Christiaens, Roger Bouillon, Hans Wildiers: Vitamin D status at breast cancer diagnosis: correlation with tumor charcteristics, disease outcome and genetic determinants of vitamin D deficiency. Carcinogenesis, accepted for publication

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Books :

-**B. Brouwers**, H. Wildiers: Book : 'Handboek kanker bij ouderen' ; Chapter on Chemotherapy in Elderly. Editor 'de Tijdstroom', The Netherlands. ISBN 978 90 5898 195 0

Abstracts :

-**Barbara Brouwers**, Robert Paridaens, Jean-Pierre Lobelle, Wouter Hendrickx, Ann Smeets, Patrick Neven, Caroline Weltens, karen Deraedt, Isabelle Vanden Bempt, Hans Wildiers: Clinicopathological features of inflammatory versus non-inflammatory locally advanced non-metastatic breast cancer. EBCC 6 (Berlin) Ejc Supplements April 2008: Abstract No 306.

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